Eosinophil activation in a mouse model of allergic airways disease

By

Kristopher Clark

Division of Molecular Biosciences
John Curtin School of Medical Research
Australian National University

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Statement of Authorship

I, Kristopher Clark, declare that the investigations described in this thesis are my own original work.

Kristopher Clark

Division of Molecular Biosciences
John Curtin School of Medical Research
Australian National University

ABSTRACT

Eosinophils are considered key effector cells in the pathogenesis of allergic diseases. In particular, eosinophils are activated at sites of inflammation to release cytotoxic cationic proteins which are thought to promote disease by inducing tissue damage and dysfunction. Indeed, eosinophil degranulation is a key feature of allergic disorders and the levels of secreted products often correlates with disease severity. Although mouse models of allergic airways disease reproduce several features of human asthma, ultrastructural evidence of active granule disassembly in mouse eosinophils has not been reported. This apparent absence of eosinophil degranulation has questionned the validity to employ this specie to model the contribution of eosinophils to the pathogenetics of allergy. In this thesis, it is demonstrated that mouse eosinophils release granule proteins by piecemeal degranulation in the allergic lung of mice. However, eosinophil degranulation is most prominent in, and primarily compartmentalized to, the airways lumen. Accordingly, recruitment and activation of eosinophils in the lung correlates with the detection of cell-free eosinophil peroxidase in BALF and with the induction of airways hyperreactivity. Furthermore, in vitro investigations revealed that higher concentrations of exogenous stimuli appear to be required to trigger both adherence and degranulation of mouse eosinophils when compared to values reported for studies of human eosinophils suggesting inter-specie differences in the threshold of eosinophil activation. Failure of eosinophils to degranulate in the allergic lung may reflect the acute nature of the inflammatory response in mice or the requirement for higher levels of stimuli to activate this cell. Thus, mouse eosinophils undergo piecemeal degranulation during allergic inflammation, and in turn, this process may contribute to pathogenesis. However, the degranulation process in the allergic lung of mice is spatially distinct from that observed in human disease.

Since the level of eosinophil activation increased as the cell migrated from the bone marrow to the airways lumen, a method which permits the purification of eosinophils from the different compartments may permit the identification of key regulators of eosinophil activation. A method to purifiy eosinophil populations from the bone marrow, blood and BALF of allergic mice was developed using FACS. The basis for the isolation method is the high SSC and light polarization properties of eosinophils. Importantly, the purified eosinophils (>96%) were viable (>95%) and responded to stimulation. Preliminary investigations highlighted the presence of several differentially expressed genes, including cytokines, cytotoxic granule proteins and chemokine and cytokine

receptors, between bone marrow and BALF eosinophils. This investigation provides a method to purify eosinophils from all compartments and subsequently, characterize the phenotypic differences between eosinophil populations.

In conclusion, mouse models of experimental asthma may provide a tool to further elucidate the mechanisms regulating eosinophil activation *in vivo*. A better understanding of mechanisms modulating eosinophil degranulation may provide a platform for the development of novel therapeutic agents for the treatment of allergic inflammatory diseases.

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ABBREVIATIONS

AHR	airways hyperreactivity
APC	antigen presenting cell
BALF	bronchoalveolar lavage fluid
BSA	bovine serum albumin
CCR	C-C chemokine receptor
cDNA	complementary DNA
Cfeg	cluster of free extracellular granules
CTAB	cetyltriethylammonium bromide
CxCR	C-x-C chemokine receptor
DAG	1,2-diacylglycerol
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
ECP	eosinophil cationic protein
EDN	eosinophil-derived neurotoxin
EDTA	ethylenediamine tetraacetic acid
ENA-78	epithelial neutrophil-activating protein 78
EPO	eosinophil peroxidase
ETE	eicosatetraenoate
FACS	fluorescence activated cell sorter
fMLP	N-formyl-methionyl-leucyl-phenylalanine
FSC	forward scatter
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	granulocyte macrophage-colony stimulating factor
GTP	guanosine triphosphate
ICAM	intercellular adhesion molecule
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IP-10	interferon-inducible protein 10
IP_3	inositol 1,4,5-triphosphate
LFA	lymphocyte function-associated antigen
LT	leukotriene
MBP	major basic protein
MCP	monocyte chemotactic protein
mEAR	mouse eosinophil-associated ribonuclease

MIP	macrophage inflammatory protein
mRNA	messenger RNA
NOS	nitric oxide synthase
OVA	ovalbumin
PAF	platelet activating factor
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
Penh	enhanced pause
pI	Isoelectric point
PI3K	phosphoinositol-3-kinase
PKC	protein kinase C
PLA2	phospholipase A2
PLC	phospholipase C
PMA	phorbol-12-myristate-13-acetate
RANTES	regulated upon activation in normal t-cells expressed and secreted
RNA	ribonucleic acid
RNase	ribonuclease
RT-PCR	reverse-transcriptase PCR
SEM	standard error of the mean
sIgA	secretory IgA
SNAP	synaptosomal-associated protein
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SSC	side scatter
t-SNARE	target SNARE
Tg	transgenic
TGF	transforming growth factor
Th	T helper cell
TNF	tumor necrosis factor
v-SNARE	vesicle SNARE
VAMP	vesicle-associated membrane protein
VCAM	vascular cell adhesion molecule
VLA	very-late antigen
WT	wild type

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return to visit one day! To all those not mentioned, I have not forgotten your contribution. THANK YOU!!!

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1

General introduction-
Eosinophil activation

1.1 INTRODUCTION

Asthma is a chronic inflammatory disease of the lower airways with a disproportionate prevalence in industrial and urban regions around the world (Busse and Lemanske, 2001; Yazdanbakhsh *et al.*, 2002). The morbidity and mortality of asthma has increased alarmingly over the last two decades highlighting the importance for the development of new therapeutic strategies (Maddox and Schwartz, 2002).

The causes of asthma are complex and multifactorial. Both a genetic predisposition and environmental factors are linked to the development of asthma (Maddox and Schwartz, 2002). Asthmatics have been divided into two groups based on the stimuli responsible for exacerbation of disease. In extrinsic asthma, sensitization to an environmental allergen leads to the production of antigen-specific immunoglobulin (Ig) E and subsequent exposure to the allergen induces clinical manifestations of disease (Bochner *et al.*, 1994). In a smaller proportion of the population, intrinsic asthma is observed whereby patients do not demonstrate elevated serum IgE levels and factors such as exercise and aspirin sensitivity initiate the onset of disease (Bochner *et al.*, 1994).

The clinical features of asthma are reversible airflow obstruction, airway histopathologies, airways hyperreactivity (AHR) to inhaled spasmogens and an underlying chronic inflammation of lower airways consisting of lymphocytes, mast cells and eosinophils (Bochner *et al.*, 1994; Busse and Lemanske, 2001; Maddox and Schwartz, 2002). Recently, the inflammatory process has been considered a key factor responsible for the pathophysiological changes associated with asthma. Inflammation of the lower airways affects airway epithelial shedding, subepithelial fibrosis, hypertrophy and hyperplasia of smooth muscle, plasma extravasation and mucus hypersecretion (Barnes, 1996b). However, the cellular composition of the pulmonary inflammation changes during the course of disease. The spatial and temporal regulation of leukocyte populations has led to two distinct periods of disease in atopic patients after allergen inhalation. The early-phase response, which occurs in the first hour, is orchestrated by mast cells whereas the late-phase response, which arises between 3 to 11 hours post-allergen provocation, is mediated by $CD4^+$ T-cells and eosinophils (Bochner *et al.*, 1994; Busse and Lemanske, 2001). The molecules that coordinate the differentiation, recruitment and activation of these leukocyte subsets are central to the pathogenesis of asthma. Since eosinophils have been associated with disease severity, elucidation of the mechanisms regulating eosinophil activation might present investigators with new targets for the treatment of allergic diseases.

1.2 CELLULAR AND MOLECULAR MECHANISMS REGULATING ALLERGIC AIRWAYS DISEASE

Clinical studies in conjunction with investigations employing animal models have identified key effector cells and molecules regulating the pathogenesis of allergic airways disease. In the current paradigm (Fig.1.1), professional antigen-presenting cells (APC), which include dendritic cells and macrophages, regulate the inflammatory process instigated by antigen provocation. At the site of allergen exposure, APCs sample the microenvironment and process foreign antigens before migrating to the draining lymph nodes where they direct T-cell differentiation towards the T-helper (Th) type 2 phenotype, and activation of T- and B-cells (Lambrecht *et al.*, 1998). Th2 lymphocytes secrete a distinct subset of cytokines including interleukin (IL)-2, IL-4, IL-5 and IL-13, which cooperate to promote eosinophilic inflammation and AHR (Webb and Foster, 1999; Webb *et al.*, 2000). IL-2 and IL-4 coordinate the amplification of the Th2 T-cell population. IL-2 provides a proliferative signal (Toribio *et al.*, 1989) while IL-4 polarizes uncommitted T-cells to the Th2 phenotype (Swain *et al.*, 1990). This local

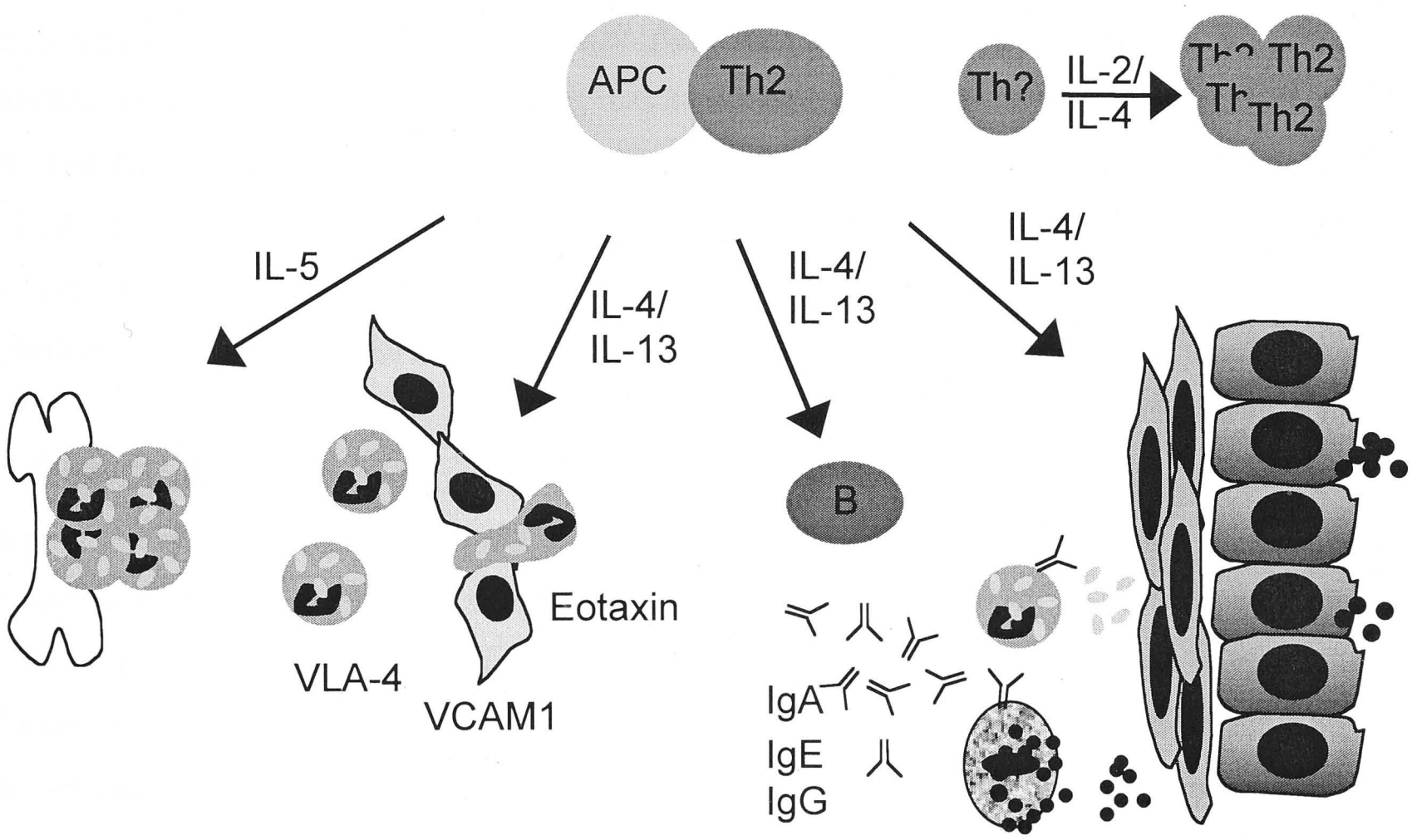


FIGURE 1.1- Cellular and molecular mechanisms regulating the pathogenesis of allergic airways disease. Upon allergen provocation, APCs activate Th2 lymphocytes which secrete various cytokines, notably IL-4, IL-5 and IL-13. The coordinate activities of these cytokines results in the generation of a blood and pulmonary eosinophilia, activation of eosinophils and potentially, the induction of AHR. (Adapted from Webb and Foster, 1999).

effect in combination with T-cell activation will lead to a systemic rise in IL-4, IL-5 and IL-13 concentrations which control eosinophil function. IL-5 directs the differentiation of eosinophil progenitors and mobilization of mature eosinophils into the bloodstream whereas IL-4 and IL-13 are implicated in eosinophil recruitment and activation (Mould *et al.*, 1997; Sanderson *et al.*, 1985). The migration of eosinophils to the allergic lung is regulated by chemotactic factors, principally members of the eotaxin family (Forssmann *et al.*, 1997; Jose *et al.*, 1994; Kitaura *et al.*, 1999; Shinkai *et al.*, 1999). Both IL-4 and IL-13 influence eosinophil movement by modulating the expression of eotaxin and adhesion systems within pulmonary tissues (Bochner *et al.*, 1995; Doucet *et al.*, 1998; Hirst *et al.*, 2002; Li *et al.*, 1999; Moore *et al.*, 2002; Schleimer *et al.*, 1992; Striz *et al.*, 1999; Terada *et al.*, 2000; Woltmann *et al.*, 2000). Eosinophils present at sites of allergic inflammation, subsequently, potentiate disease processes such as AHR through the release of pro-inflammatory mediators (Gundel *et al.*, 1991; Uchida *et al.*, 1993). This latter process may also be influenced by both IL-4 and IL-13 since these cytokines are essential for isotype switching of B-cells (Punnonen *et al.*, 1993) and immunoglobulins represent a key stimulus for the activation of eosinophil secretory pathways (Abu-Ghazaleh *et al.*, 1989; Kaneko *et al.*, 1995b; Khalife *et al.*, 1986; Khalife *et al.*, 1985). Finally, IL-4 and IL-13 can directly affect the induction of AHR and mucus hypersecretion without the requirement of additional participants (Grunig *et al.*, 1998; Venkayya *et al.*, 2002; Wills-Karp *et al.*, 1998; Yang *et al.*, 2001). Hence, the late-phase response associated with asthma arises through the coordinate actions of various cytokines on different leukocyte populations.

1.3 EOSINOPHILS

Eosinophils are granulocytes derived from myeloid progenitors resident in the bone marrow with a dramatic expansion of the eosinophil population in various disorders including parasite infections, atopic disease and cancer (Rothenberg, 1998). Eosinophils have a protective role against parasite infestations but a pro-inflammatory effect during allergic diseases. Indeed, eosinophils are considered key effector cells in the pathogenesis of atopic disorders affecting the airway and gastrointestinal mucosa (Hogan *et al.*, 2002; Rothenberg, 1998; Wardlaw *et al.*, 1995).

Ultrastructural analyses of human eosinophils have revealed several features (Fig.1.2)- nuclei are polylobed with partially condensed chromatin, irregular surface projections and the presence of various secretory granules (Dvorak, 1994; Dvorak *et al.*, 1993a). Based on granule content, three granule types have been identified. Primary granules are membrane-bound spherical structures with a homogenous content. These granules

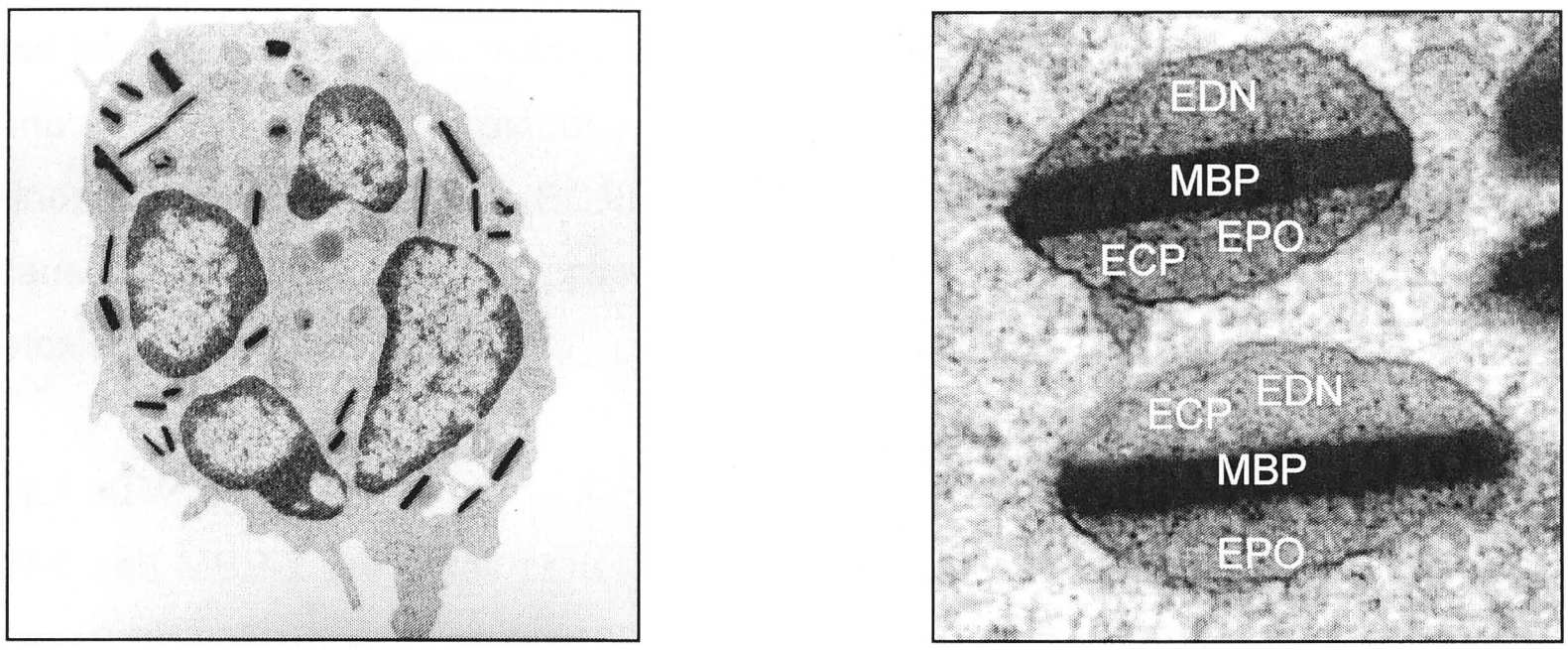


FIGURE 1.2- Electron photomicrographs of an eosinophil and its secondary granules. *A.* Ultrastructural features of eosinophils include irregular surface projections, a polylobed nucleus and various secretory granules notably secondary granules. *B.* The secondary granule is a spherical, membrane-bound structure, which can be recognized by its electron-dense crystal core surrounded by a matrix. These granules are storage sites for the cytotoxic cationic proteins and other mediators such as cytokines and chemokines.

are the primary storage site of Charcot-Leyden crystal protein (Dvorak *et al.*, 1988). The secondary granule (or specific granule) is also a membrane-bound spherical structure but easily distinguishable from other granules since it consists of an electron-dense crystalloid core surrounded by a less dense matrix. These granules contain the cytotoxic cationic proteins as well as several cytokines and chemokines (Egesten *et al.*, 2001). Other visible structures include the small granules which contain hydrolytic enzymes (Egesten *et al.*, 2001) and lipid bodies which consists mainly of arachidonic acid and its metabolic products (Bandeira-Melo *et al.*, 2002).

The role of eosinophils in both parasite clearance and pathogenesis of allergic diseases is attributable to the release of pro-inflammatory molecules which include the cytotoxic cationic granule proteins, lipid mediators, reactive oxygen species, cytokines and chemokines. The effect of these mediators in regulating physiological processes has been partially defined and will be briefly summarized.

1.3.1 Cytotoxic cationic granule proteins

Human eosinophils are the primary source of a class of pro-inflammatory molecules called the cytotoxic cationic granule proteins, which include major basic proteins (MBP-1 and -2), eosinophil peroxidase (EPO), eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN). The proteins have been classified into this family because they share several properties notably, a high isoelectric point (pI) and cytotoxicity towards parasites and mammalian cells.

1.3.1.1 MBP

MBP-1 and MBP-2 are small proteins of approximately 15 kDa which are highly basic (pI = 10.9) (Wasmoen *et al.*, 1988; Plager *et al.*, 1999). However, it has been hypothesized that to protect the eosinophil, MBP-1 is translated as a prepropeptide whereby the amino terminal sequence targets the protein to the appropriate cellular compartment and the propeptide which is highly acidic (pI=3.9) neutralizes the toxicity of MBP-1 (Popken-Harris *et al.*, 1998). The mature MBP-1 product is stored in secondary granules where it forms the crystal core (Peters *et al.*, 1986). MBP-2 displays similar properties to MBP-1 (Macias *et al.*, 2000; Plager *et al.*, 1999). However, in the absence of MBP-1, no crystal structure is formed in the secondary granules suggesting that MBP-2 alone is incapable of forming this structure (Denzler *et al.*, 2000).

Although MBP-1 has no enzymatic activity, it has been directly implicated in the clearance of parasites and pathogenesis of allergic diseases. *In vitro* experiments have demonstrated the potential of MBP-1 to kill parasites and bacteria (Butterworth *et al.*, 1979; Hamann *et al.*, 1987; Hamann *et al.*, 1990; Lehrer *et al.*, 1989). Furthermore, the cytotoxic nature of MBP-1 is extended to several mammalian cells including airway epithelial cells (Ayars *et al.*, 1989; Frigas *et al.*, 1980; Hisamatsu *et al.*, 1990). MBP-1 may also contribute to the pathogenesis of allergic airways disease through the induction of AHR. In particular, MBP-1 induces AHR when instilled into the airways of guinea pigs and primates by inhibiting M_2 muscarinic receptor function which leads to an increase in acetylcholine concentration and to a subsequent increase in vagally induced bronchoconstriction (Gundel *et al.*, 1991; Jacoby *et al.*, 1993; Uchida *et al.*, 1993). Finally, MBP-1 can affect disease processes through its immunoregulatory functions. MBP-1 can activate secretory pathways in platelets, mast cells, basophils, neutrophils and eosinophils in a noncytolytic mechanism (Kita *et al.*, 1995; Moy *et al.*, 1990; O'Donnell *et al.*, 1983; Rohrbach *et al.*, 1990; Zheutlin *et al.*, 1984).

1.3.1.2 EPO

EPO is the largest cationic granule protein composed of an 11 kDa and a 57 kDa subunit in conjunction with a heme-moeity (Ten *et al.*, 1989). The products of reactions catalyzed by EPO have been associated with the pathogenesis of asthma and cancer as well as host defense against parasite and helminth infections. Although substrates are not required for EPO-mediated killing of parasites, H_2O_2 dramatically enhanced the cytotoxicity of EPO (Hamann *et al.*, 1990; Weiss *et al.*, 1986).

EPO utilizes halides (Br^-, Cl^-, I^-) and pseudohalides (SCN^-), but preferentially bromide and thiocyanate, in combination with H_2O_2 to generate the corresponding hypohalous acids (van Dalen and Kettle, 2001). This reaction has been implicated in the bromination of tyrosine residues found in proteins located in the asthmatic lung (Wu *et al.*, 1999a; Wu *et al.*, 2000) and in the pathogenesis of cancer through the production of mutagenic bromonucleotides and oxidative damage of deoxyribonucleic acid (DNA) (Henderson *et al.*, 2001a; Henderson *et al.*, 2001b; Shen *et al.*, 2001). Alternatively, the hypohalous acid can combine with superoxide to form the highly reactive hydroxyl radical which promotes mutagenesis through nucleotide and DNA modifications (Shen *et al.*, 2000).

Another mechanism for the oxidation of proteins catalyzed by EPO is through the H_2O_2 /NO_2 pathway which results in the nitration of tyrosyl residues in proteins (Brennan *et al.*, 2002; Wu *et al.*, 1999b). 3-nitrotyrosine, a marker of protein nitration, is elevated in the asthmatic lung (MacPherson *et al.*, 2001). Moreover, recent evidence suggests that protein nitration is eosinophil- and EPO-dependent (Brennan *et al.*, 2002; Iijima *et al.*, 2001). Although EPO can modify DNA and proteins by various mechanisms, the pathological significance of the products has yet to be determined.

1.3.1.3 ECP and EDN

ECP and EDN are small basic proteins found in the matrix of secondary granules. ECP is also toxic towards helminthes, parasites, bacteria and mammalian cellular targets (Ackerman *et al.*, 1985; Hamann *et al.*, 1987; Hamann *et al.*, 1990; Lehrer *et al.*, 1989; Motojima *et al.*, 1989). Although EDN can mediate the killing of parasites, it is less potent than the other cationic toxins (Ackerman *et al.*, 1985; Hamann *et al.*, 1987; Hamann *et al.*, 1990). In contrast to MBP-1 and EPO, both ECP and EDN could not induce bronchoconstriction in primates (Gundel *et al.*, 1991). However, ECP and EDN have been associated with the pathogenesis of the Gordon phenomenon which is a disorder afflicting the nervous system (Fredens *et al.*, 1982).

The distinct roles of ECP and EDN in disease processes may be due to their particular properties. ECP and EDN are members of the ribonuclease (RNase) A superfamily (Barker *et al.*, 1989; Rosenberg *et al.*, 1989a; Rosenberg *et al.*, 1989b) and thus, possess RNase activity with EDN being 100-fold more active than ECP (Slifman *et al.*, 1986). Mouse eosinophils express a set of 15 genes named eosinophil-associated ribonucleases (mEAR) which are the paralogues of the ECP and EDN genes of primates (Rosenberg and Domachowske, 2001). Eosinophils have been traditionally associated with Th2 type immunological responses. However, emerging evidence suggests that eosinophils, through the coordinate action of the RNases, may play a role in host defense against viral pathogens (Domachowske *et al.*, 1998a; Domachowske *et al.*, 1998b; Rosenberg and Domachowske, 2001).

1.3.2 Lipid bodies

Lipid bodies were identified in human eosinophils by electron microscopy as an electron-dense, non-membrane bound, spherical structure within the cytoplasm (Weller and Dvorak, 1985). Lipid bodies are composed of arachidonic acid principally in the esterified form (Weller and Dvorak, 1985; Weller *et al.*, 1991), enzymes required for the metabolism of this lipid (Bozza *et al.*, 1997; Dvorak *et al.*, 1994) and signaling molecules (Yu *et al.*, 1998). Immunolocalization studies have demonstrated the presence of the eicosanoid-forming enzymes which include 5-lipoxygenase, leukotriene (LT) C4 synthase and cyclooxygenases (Bozza *et al.*, 1997; Dvorak *et al.*, 1994). The transmission of extracellular signals for the activation of eicosanoid-forming enzymes occurs through phospholipase A2 (PLA2) and MAP kinases including ERK1, ERK2 and p38 present in lipid bodies (Yu *et al.*, 1998). Thus, lipid bodies contain all the components to behave as a separate entity for the regulation of eicosanoid production and release.

The formation of lipid bodies is a characteristic feature of human eosinophil activation. Only a few lipid bodies are found in normal peripheral blood eosinophils whereas numerous lipid bodies may be found in activated eosinophils (Bozza *et al.*, 1996; Bozza *et al.*, 1998; Bozza *et al.*, 1997; Weller and Dvorak, 1985). Activation human of eosinophils leads not only to increased numbers of lipid bodies but also *de novo* synthesis of metabolic enzymes and arachidonic acid metabolism (Bozza *et al.*, 1996; Bozza *et al.*, 1997). The production of lipid mediators and subsequent release at sites of inflammation may modulate disease processes underlying asthma.

1.3.3 Reactive oxygen and nitrogen species

Activation of eosinophils results in the production of both reactive oxygen and nitrogen species. However, the proteins involved in the generation of these oxidants are distinct. NADPH oxidases are composed of seven subunits and spatial regulation of these proteins modulates enzymatic activity (Babior, 1999). A complex of five proteins is localized in the cytoplasm which translocates to the membrane upon cellular activation where it associates with cytochrome b_{558} to form an active complex (Babior, 1999). NADPH oxidases catalyze the reduction of oxygen using NADPH to produce superoxide, which is highly reactive and can dismutate to form H_2O_2, an important substrate of peroxidases (Babior, 1978a; Babior, 1978b). The oxidants kill parasites but may also harm the surrounding tissues (Babior, 1978a; Babior, 1978b; Henderson *et al.*, 2001a; Henderson *et al.*, 2001b; Shen *et al.*, 2001; Shen *et al.*, 2000).

Reactive nitrogen species are generated by several cell types expressing specific isoforms of nitric oxide synthase (NOS). Leukocytes including eosinophils express inducible-NOS which converts L-arginine to citrulline and NO in a redox reaction (Folkerts *et al.*, 2001). The concentration of nitric oxide is increased in the exhaled air of asthmatics and current evidence suggests that eosinophils are the primary source (Barnes, 1996a; Iijima *et al.*, 2001). The potential fate of NO is dependent on its environment since it can react with several molecules including oxygen and superoxide or consumed by enzymes such as EPO (Abu-Soud *et al.*, 2001; Beckman and Koppenol, 1996). A principal product of NO chemistry is the nitration of tyrosine residues in proteins. Within the asthmatic lung, protein nitration is elevated due to the catalytic activity of EPO (Brennan *et al.*, 2002; Duguet *et al.*, 2001). Unfortunately, the contribution of NO to the pathogenesis of allergic airways disease remains unclear. Inhibition of NOS had minor effects on the early and late-phase responses in atopic patients (Taylor *et al.*, 1998). However, both *in vitro* and *in vivo* experiments suggest that NO may regulate the recruitment of inflammatory cells (De Sanctis *et al.*, 1999; Thomazzi *et al.*, 2001; Xiong *et al.*, 1999). The spatial and temporal regulation of NO might be an important aspect during allergic reactions since different NOS isoforms have distinct effects on the pathogenesis of disease. Studies using genetically-modified mice have revealed an important role for neuronal isoforms of NOS in modulating AHR whereas AHR in inducible-NOS knockout mice was not significantly different to wild-type (WT) mice although, inflammation was reduced (De Sanctis *et al.*, 1999).

1.3.4 Cytokines and chemokines

Both human and mouse eosinophils are sources of Th1 and Th2 cytokines including IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, IL-16, IL-18, granulocyte macrophage-colony stimulating factor (GM-CSF), tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-α and TGF-β(Egesten *et al.*, 2001; Mattes *et al.*, 2002; Schmid-Grendelmeier *et al.*, 2002; Woerly *et al.*, 1999).The proteins have a wide-range of regulatory functions which permit eosinophils to exert an influence on the development of both humoral and cellular immune responses. Eosinophils also express chemokines which may amplify eosinophilic inflammation. In particular, eosinophils synthesize eotaxin, regulated upon activation in normal T-cells expressed and secreted (RANTES), macrophage inflammatory protein (MIP)-1α and IL-8 (Egesten *et al.*, 2001).

1.4 ROLE OF EOSINOPHILS IN ALLERGIC AIRWAYS DISEASE

The role of eosinophils in the pathogenesis of allergic airways disease remains controversial. Over the past decades, several clinical studies have demonstrated a strong correlation between eosinophilic inflammation, exacerbation of disease and disease severity (Bradley *et al.*, 1991; Fujimoto *et al.*, 1997; Louis *et al.*, 2000; Reed, 1994). Importantly, activated eosinophils were present at sites of inflammation, which was associated with an extensive release of granule proteins in the lower airways of asthmatics (Filley *et al.*, 1982). Eosinophils have also been localized in close proximity to nerves and smooth muscle within the allergic lung (Evans *et al.*, 2001). The spatial regulation of eosinophils (Evans *et al.*, 2001) and the ability of MBP-1 and EPO to modulate AHR through the allosteric antagonism of inhibitory M2 muscarinic receptors (Jacoby *et al.*, 1993) suggests a pro-inflammatory role for eosinophils in the pathogenesis of allergic airways disease.

Studies employing animal models of allergic airways disease have supported these concepts. The kinetics for the development of a pulmonary eosinophilia in response to aeroallergen challenge correlates with the onset of AHR (Tomkinson *et al.*, 2001). Furthermore, depletion of IL-5 results in a dramatic reduction in eosinophil recruitment to the allergic lung, which accompanies an improvement in various disease parameters (Foster *et al.*, 1996; Hamelmann *et al.*, 1999a). Finally, neutralization of eosinophil by-products, notably MBP-1, inhibited antigen-induced AHR (Lefort *et al.*, 1996).

However, other studies dispute this role of eosinophils in atopic diseases. Certain clinical studies have failed to correlate eosinophil numbers and AHR (Crimi *et al.*, 1998). Moreover, the administration of a humanized IL-5 monoclonal antibody to asthmatics improved disease processes marginally although it significantly reduced blood and pulmonary eosinophilia (Leckie *et al.*, 2000). An IL-5 independent mechanism for the induction of AHR, which has been interpreted as an eosinophil-independent mechanism, has also been described in experimental models of asthma (Corry *et al.*, 1996; Hessel *et al.*, 1997; Hogan *et al.*, 1998). In addition, eosinophil degranulation, a hallmark feature of human asthma, does not occur in the submucosa of the allergic lung in mice (Malm-Erjefalt *et al.*, 2001; Stelts *et al.*, 1998). These findings have led to the suggestion that eosinophils may not regulate certain disease processes such as AHR and mucus hypersecretion in experimental models and thus by inference, allergic asthma.

Clearly, eosinophils possess the potential to play a critical role however, additional investigations must be undertaken to determine the precise role of eosinophilic inflammation in the pathogenesis of allergic airways disease.

1.5 MECHANISMS OF HUMAN EOSINOPHIL DEGRANULATION

Eosinophils are secretory cells which store pro-inflammatory mediators in membrane-bound cytoplasmic granules. The release of the cytotoxic cationic proteins, termed degranulation, into the micro-environment may occur via three mechanisms namely exocytosis, piecemeal degranulation and cytolysis (Fig.1.3). Electrophysiological measurements of activated eosinophils have provided support for the classical exocytosis mechanism (Lindau *et al.*, 1994; Lindau *et al.*, 1993) whereas electron microscopic analysis of eosinophils has revealed the occurrence of piecemeal degranulation and cytolysis (Dvorak *et al.*, 1992; Dvorak *et al.*, 1991; Erjefalt *et al.*, 1998; Persson and Erjefalt, 1997).

1.5.1 Classical exocytosis

Secretion by exocytosis involves fusion of the membrane enveloping the secondary granule with the plasma membrane. This process results in an increase in plasma membrane surface area which is directly proportional to a subsequent change in plasma membrane capacitance. By time-resolved patch-clamp capacitance measurements, Nusse and colleagues (Nusse *et al.*, 1990) demonstrated that eosinophils secreted secondary granule contents through an exocytotic mechanism

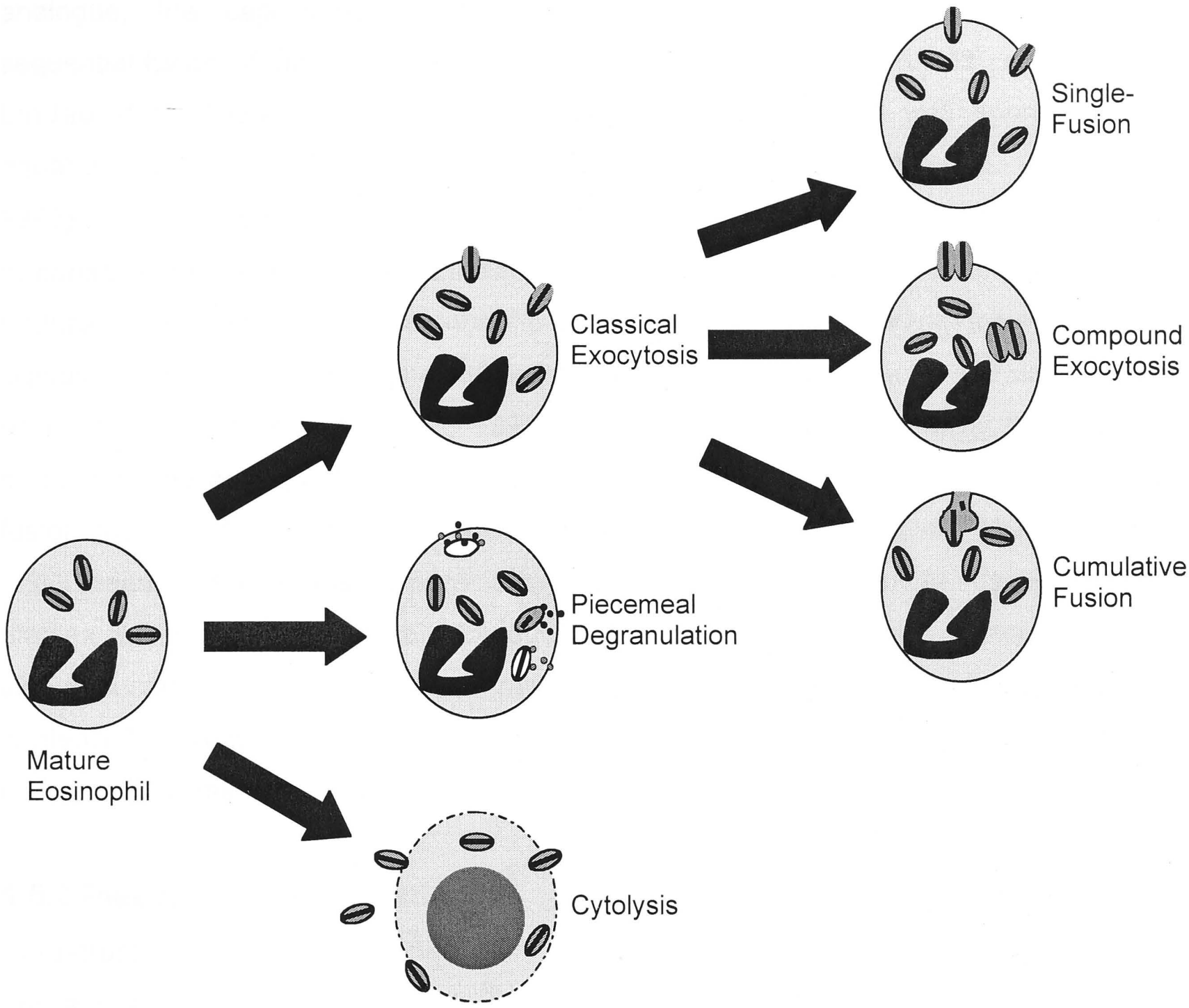


FIGURE 1.3- Mechanisms of human eosinophil degranulation. The release of the cytotoxic cationic proteins may occur via classical exocytosis, piecemeal degranulation or cytolysis. Classical exocytosis is a secretory process whereby the membrane of the secondary granule fuses with the plasma membrane resulting in the release of granule contents. In addition to single-fusion events, secondary granules may fuse within the cytoplasm before release of the granule contents in a process termed compound exocytosis and spatial regulation of the secretory process has led to the observation of cumulative fusion whereby successive fusion events occur at the initial fusion pore. During piecemeal degranulation, small vesicles bud from the secondary granule carrying a subset or complete set of mediators which are transported to the cell surface to be secreted. Cytolysis is the release of all cellular contents including secondary granules upon the rupture of the plasma membrane.

upon guanosine triphosphate (GTP)-γ-S stimulation. At low concentrations of the GTP analogue, the capacitance of cells increased stepwise in accordance with the sequential fusion of individual granules with the plasma membrane (Lindau *et al.*, 1994; Lindau *et al.*, 1993). Furthermore, the number of fusion events was approximately equal to the number of secondary granules contained in a resting cell (Lindau *et al.*, 1993). When the concentration of GTP-γ-S was increased, fewer but larger increases in capacitance were recorded (Lindau *et al.*, 1994; Scepek and Lindau, 1993). This feature is characteristic of eosinophils undergoing compound exocytosis whereby granules fuse together within the cell before transport to the plasma membrane (Lindau *et al.*, 1994; Scepek and Lindau, 1993). Finally, eosinophils may target the granule proteins to the appropriate extracellular surfaces through a process called cumulative fusion requiring the formation of a degranulation sac (Newman *et al.*, 1996; Scepek and Lindau, 1993). A fusion pore is created when the first granule fuses with the plasma membrane and directs subsequent fusion events at this site. Although few *in vivo* observations of this mechanism have been reported, eosinophils released granule contents by exocytosis in the gut of patients suffering from tissue-invasive bacterial infections (Dvorak *et al.*, 1993b).

1.5.2 Piecemeal degranulation

Ultrastructural analysis of human eosinophils revealed the occurrence of piecemeal degranulation which is characterized by the gradual emptying of the crystalloid containing granules (Dvorak *et al.*, 1992; Dvorak *et al.*, 1991). Upon activation of eosinophils, small vesicles bud from the secondary granules carrying a subset or complete set of mediators and deliver their contents to the extracellular space by exocytosis (Dvorak *et al.*, 1992; Dvorak *et al.*, 1991). This mechanism permits the secretion of specific granule proteins (Tomassini *et al.*, 1991; Torpier *et al.*, 1988). Importantly, the nature of the stimuli determines the identity of the granule proteins secreted by the eosinophil (Tomassini *et al.*, 1991). In contrast to exocytosis, piecemeal degranulation has been widely observed in both cultured cells and in eosinophils present in inflammed tissues (Dvorak, 1994; Dvorak *et al.*, 1992; Dvorak *et al.*, 1991; Erjefalt *et al.*, 1998; Erjefalt *et al.*, 1999).

1.5.3 Cytolysis

The presence of cytolytic eosinophils and their by-products have been described in various disorders associated with an eosinophilia which is consistent with the frequent observation that tissues afflicted by high levels of eosinophil degranulation present only few intact eosinophils (Wardlaw *et al.*, 1995). Notably, cytolysis is the primary

mechanism of degranulation in the skin of patients suffering from hypereosinophilic syndrome and bullous pemphigoid (Dvorak, 1994). In these diseases, cytolysis is characterized by centralization of granules, chromatolysis within the nuclei and loss of plasma membrane integrity (Erjefalt *et al.*, 1998). As a result, the entire cellular contents of eosinophils are released into the extracellular matrix and clusters of free extracellular granules (Cfegs) are generated. Previously, this mechanism of degranulation was regarded as a passive form of granule release induced by cell injury. However, emerging evidence suggests that cytolysis may be actively regulated by the eosinophil and serve as the ultimate form of eosinophil activation (Erjefalt *et al.*, 1998; Erjefalt *et al.*, 1999).

1.5.4 Eosinophil degranulation in the airways

Although the release of granule proteins in the airways of asthmatic patients has been observed for several decades, the mechanism of eosinophil degranulation has been investigated only recently. Ultrastructural analysis of eosinophils present in the airways of patients suffering from allergic rhinitis show that the majority of eosinophils released granule contents by piecemeal degranulation with a significant proportion of eosinophils undergoing cytolysis (Erjefalt *et al.*, 1998; Erjefalt *et al.*, 1999). Although fewer eosinophils were cytolytic, these cells may contribute the greatest proportion towards the cytotoxic granule protein concentration within the airways.

In summary, eosinophils can utilize classical exocytosis, piecemeal degranulation or cytolysis to release the contents of secondary granules. However, the extracellular stimuli and their intracellular signaling pathways regulating these 3 processes have yet to be defined. It will be interesting to determine how the mechanisms of eosinophil degranulation are differentially regulated both by the nature of the stimuli and the specific intracellular cascades.

1.6 ACTIVATION OF HUMAN EOSINOPHILS

The cellular functions of eosinophils are regulated by the activation status of the cell. Several changes in phenotype upon activation of human eosinophils have been observed and include a decrease in cell density, differential expression of cell surface markers and enhanced adhesion, respiratory burst, bioactive lipid release and degranulation (Busse *et al.*, 1996; Carlson *et al.*, 1993; Takafuji *et al.*, 1991; Takafuji *et al.*, 1996). Although the mechanisms which regulate human eosinophil degranulation are complex, the process may be divided into two distinct steps: 1) priming of eosinophils predisposes the cell to generate a specific response without the priming

agent itself inducing the response and 2) activation of eosinophil degranulation by a secretagogue leading to the release of cytotoxic granule proteins. The priming event is required for optimal release of granule proteins. Dissection of the role of molecules involved in eosinophilic inflammation suggest that cytokines and chemokines are primarily priming agents (Alam *et al.*, 1993; Carlson *et al.*, 1993; Fujisawa *et al.*, 1990; Rot *et al.*, 1992; Takafuji *et al.*, 1996) whereas immunoglobulins, complement fragments and lipid mediators are secretagogues (Abu-Ghazaleh *et al.*, 1989; Kaneko *et al.*, 1995b; Khalife *et al.*, 1986; Khalife *et al.*, 1985; Kroegel *et al.*, 1988; Kroegel *et al.*, 1989; Takafuji *et al.*, 1994; Takafuji *et al.*, 1998). The engagement of adhesion systems is an essential co-stimulatory stimulus (Horie and Kita, 1994; Kaneko *et al.*, 1995a) and will be a recurring theme.

1.6.1 Cytokines

Cytokines regulate the differentiation, migration and activation of leukocytes. Various studies have demonstrated a role for both Th1 and Th2 cytokines in the regulation of eosinophil activation. While certain cytokines served as a direct degranulation signal, others had a priming effect and some cytokines had an inhibitory effect on eosinophil degranulation.

1.6.1.1 IL-5

Eosinophil biology has focused attention towards the cytokines IL-3, IL-5 and GM-CSF because these molecules are implicated in the maturation of eosinophil progenitors and provide survival signals for eosinophils. These cytokines are also associated with Th2 immunity which regulates eosinophilia and the allergic response. IL-5 may serve as both a factor which primes human eosinophils for degranulation and as a secretagogue. Pre-incubation with IL-5 enhanced, in a dose- and time-dependent mechanism, the immunoglobulin-induced degranulation of eosinophils (Fujisawa *et al.*, 1990; Kita *et al.*, 1992). A similar effect was observed when monitoring the release of ECP after stimulation with the complement proteins C3a, C3b and C5a (Carlson *et al.*, 1993; Takafuji *et al.*, 1996). The mechanism of IL-5 priming may be to enhance eosinophil adhesion which activates signaling pathways regulating the degranulation process (Fujisawa *et al.*, 1997; Kato *et al.*, 1998a). The role of IL-5 in modulating granulocyte degranulation is specific for eosinophils because neutrophil degranulation as measured by the release of myeloperoxidase and lactoferrin is unaffected by IL-5 pre-treatment (Carlson *et al.*, 1993). Thus, a potential mechanism exists at sites of inflammation to regulate the degranulation of specific granulocyte populations.

The priming of eosinophil degranulation by IL-5 is a very rapid response. During this period, IL-5 is unable to serve as a secretagogue. However, chronic exposure of human eosinophils to IL-5 leads to the release of granule proteins. After 3 days in culture medium supplemented with IL-5, eosinophils had secreted significant amounts of all granule proteins with only 59% of the total MBP remaining in the granules (Kita *et al.*, 1992). Additional degranulation of eosinophils occurred up to day 7. Importantly, the morphology of the eosinophils was characteristic of activated eosinophils present in inflammatory disorders. Although IL-5 can induce the secretion of granule proteins, the kinetics of this process and the potency of IL-5 suggest that the principal role of this cytokine in relation to degranulation at sites of inflammation is to enhance the response of eosinophils to other stimuli (Fujisawa *et al.*, 1997; Horie *et al.*, 1996; Kita *et al.*, 1992). Furthermore, transgenic mice overexpressing IL-5 have a normal phenotype aside from the accentuated eosinophilia (Dent *et al.*, 1990). This *in vivo* observation supports the notion that additional signals (to IL-5) are required for eosinophil degranulation.

1.6.1.2 IL-3 and GM-CSF

Both IL-3 and GM-CSF enhanced the degranulation of human eosinophils in response to different stimuli. IL-3 synergized with C3a and C5a to induce ECP release from eosinophils (Takafuji *et al.*, 1996). However, in contrast to IL-5, IL-3 had a marginal effect on C3b-induced eosinophil degranulation (Carlson *et al.*, 1993). This differential priming effect of IL-3 and IL-5 suggests that priming agents regulate separate signaling pathways (further discussed in section 1.6.8.1- Cytokine priming: IL-4 and IL-5 signaling pathways). The degranulation of eosinophils in response to immunoglobulins was enhanced by priming eosinophils with either IL-3 or GM-CSF (Fujisawa *et al.*, 1990). Notably, GM-CSF was the only one of the three cytokines which demonstrated a potent secretagogue effect on human eosinophils (Carlson *et al.*, 1993; Fujisawa *et al.*, 1990). The efficiency of GM-CSF is similar to secretory (s)IgA complexed to beads and platelet activating factor (PAF) which are among the most potent degranulation stimuli reported (Horie *et al.*, 1996). Furthermore, GM-CSF required the adhesion of eosinophils via a β2-integrin dependent mechanism to induce eosinophil degranulation (Horie and Kita, 1994).

1.6.1.3 IL-4 and IL-13

IL-4 and IL-13 have been directly implicated in the regulation of immunological processes underlying the asthmatic phenotype. These cytokines possess common functions as they share the IL-4Rα receptor subunit. Effects on T- and B-lymphocyte

biology, endothelial cells and AHR are well documented (Bochner *et al.*, 1995; Grunig *et al.*, 1998; Punnonen *et al.*, 1993; Schleimer *et al.*, 1992; Swain *et al.*, 1990; Venkayya *et al.*, 2002; Wills-Karp *et al.*, 1998; Yang *et al.*, 2001). However, investigations focusing on the role of IL-4 and IL-13 in directly regulating eosinophil degranulation are limited. A short exposure (1 hr) of human eosinophils to IL-4 had no or little effect on immunoglobulin-dependent degranulation. However, a longer exposure led to the down-regulation of IgG receptors along with a corresponding decrease in IgG-mediated degranulation (Baskar *et al.*, 1990). The effect of IL-4 on FcR is specific for the IgG receptors as both the expression of IgE receptors and its modulation of eosinophil degranulation remained intact after IL-4 treatment (Baskar *et al.*, 1990). Independent investigations monitoring the effect of IL-4 on human eosinophil rosette formation on IgG- and IgA- coated beads confirmed that transient exposure to IL-4 has little effect on FcγR activation. However, IL-4 can stimulate IgA binding to the FcαR on the surface receptors (Bracke *et al.*, 1997). Since IgA is a potent eosinophil secretagogue (Abu-Ghazaleh *et al.*, 1989), IL-4 may prime IgA-mediated eosinophil degranulation. However, this hypothesis has yet to be tested. IL-13 has also been implicated in the regulation of eosinophil activation (Luttmann *et al.*, 1996). However, whether IL-13 can also regulate eosinophil degranulation remains unresolved.

1.6.1.4 TNF-α

TNF-α is another cytokine which is up-regulated during allergic diseases. The effects of TNF-α on the pathogenesis of disease may rely on its modulation of eosinophil activation. Although TNF-α did not directly induce degranulation, it enhanced the stimulatory effects of IL-5 and serum opsonized particles on human eosinophil secretory pathways (Egesten *et al.*, 1998).

1.6.1.5 TGF-β1 and IFN-γ

While certain cytokines may prime eosinophil function, other cytokines may serve as negative regulators of eosinophil degranulation. Certain cytokines expressed during a Th1 immune response serve to suppress Th2 driven immunological networks. Th1 cytokines with an inhibitory role in eosinophil degranulation include TGF-β1 and interferon (IFN)-γ. The latter down regulated the secretion of EDN from sIgA activated human eosinophils (Fujisawa *et al.*, 1990).

1.6.1.6 IL-1β

Another class of cytokines exists whereby the molecules have a dual role in enhancing and suppressing eosinophil secretory pathways. While IL-1β had no effect on EDN release, it inhibited the release of β-glucuronidase and arylsulfatase human eosinophils stimulated with IgG but enhanced the secretion of these enzymes after IgE stimulation (Baskar and Pincus, 1992).

1.6.1.7 Other cytokines

Additional cytokines produced during a Th1 and Th2 immune response have been analyzed for their effects on human eosinophil degranulation. A number of cytokines had neither an enhancing nor a suppressing effect on degranulation. IL-2, IL-6, and IL-8 had no effect on eosinophil degranulation (Fujisawa *et al.*, 1990; Horie *et al.*, 1996).

In summary, the effects of cytokines on eosinophil activation are complex. While certain cytokines may influence the pathogenesis of allergic diseases via the regulation of eosinophil effector functions, other cytokines regulate disease processes in an eosinophil-independent mechanism.

1.6.2 Chemokines

The principal function of chemokines is to attract circulating leukocytes into tissues and subsequently, direct the migration of the cells to sites of inflammation. A number of chemokines which regulate the migration of eosinophils to mucosal surfaces have been identified. Interestingly, a subset of these chemokines may also modulate eosinophil effector functions.

Chemokines are classified principally into two families called the C-C chemokines where the two cysteine residues are adjacent while the C-x-C chemokines have a single amino acid separating the two cysteine residues (Baggiolini and Dahinden, 1994). Members of the C-x-C family include IL-8, MIP-2, epithelial neutrophil-activating protein (ENA)-78 and interferon-inducible protein (IP)-10. These molecules affect mainly the function of neutrophils. It is the C-C chemokines which have sustained the greatest interest in studies of diseases associated with an eosinophilia because of their effect on eosinophil functions (Rothenberg *et al.*, 1999). RANTES, eotaxin-1, eotaxin-2, eotaxin-3, MIP-1α, monocyte chemotactic protein (MCP)-2, MCP-3 and MCP-4 are all C-C chemokines.

To date, only a few studies have reported any effect of chemokines on eosinophil activation with a focus on the up-regulation of CD11b expression and/or oxygen radical production. Specific chemokines such as eotaxin-1 and -2 and RANTES have been shown to induce a respiratory burst in human eosinophils (Elsner *et al.*, 1998; Rot *et al.*, 1992; Tenscher *et al.*, 1996). With regards to eosinophil degranulation, eotaxin-1, RANTES and MIP-1α induce the release of cytotoxic granule proteins by human eosinophils whereas other chemokines such as MIP-1β, MCP-1 and IL-8 were ineffective in causing the release of ECP (Alam *et al.*, 1993; El-Shazly *et al.*, 1998; Horie *et al.*, 1996; Rot *et al.*, 1992). However, the mechanism by which chemokines regulate eosinophil effector functions remains poorly defined.

1.6.3 Immunoglobulins

Elimination of a parasitic infestation is associated with the production of antigen-specific immunoglobulins and an eosinophil-rich inflammatory response. Since the opsonized particle is non-phagocytosable, other effector mechanisms are required for the removal of parasites. *In vitro* experiments demonstrated that eosinophils can secrete cytotoxic molecules including the cationic proteins on the surface of parasites (Butterworth, 1984). Hence, the stimulus inducing the release of the cytotoxic mediators was hypothesized to be immunoglobulins, which interact with specific Fc receptors at the surface of eosinophils.

1.6.3.1 IgG, IgA and sIgA

Several laboratories have analyzed the efficacy of a large number of antibody isotypes in activating human eosinophil degranulation. Although the antibody isotypes IgM and IgD had little effect on eosinophil effector functions, IgA, sIgA and IgG induced the release of granule contents by eosinophils (Abu-Ghazaleh *et al.*, 1989; Kaneko *et al.*, 1995b; Khalife *et al.*, 1985). The immunoglobulin-induced eosinophil degranulation may be enhanced by priming factors and is dependent on the ligation of the β2 integrin, Mac-1(Fujisawa *et al.*, 1990; Kaneko *et al.*, 1995a; Kita *et al.*, 1992).

Interestingly, the most potent immunoglobulin was sIgA (Abu-Ghazaleh *et al.*, 1989). The secretory component is crucial for the difference in potency observed with IgA and IgG (Motegi and Kita, 1998). While the secretory fragment has little effect on its own, it acts synergistically with other agonists such as cytokines and immunoglobulins to induce eosinophil degranulation (Motegi and Kita, 1998).

1.6.3.2 IgE

Atopic diseases are characterized by elevated levels of both total and antigen-specific IgE antibodies, which correlate with disease severity (Sears *et al.*, 1991). The principal role of IgE is the activation of histamine release from mast cells and basophils (Galli *et al.*, 1991). IgE may also affect disease processes by regulating eosinophil function since human eosinophils express IgE receptors including the low- (FcεRII) and high-affinity (FcεRI) IgE receptors as well as another IgE binding molecule, Mac-2 (Capron *et al.*, 1986; Gounni *et al.*, 1994; Grangette *et al.*, 1989; Truong *et al.*, 1993). Moreover, IgE levels are associated with eosinophilia and FcεRI positive eosinophils are present in the tissues of atopic patients (Barata *et al.*, 1997; Rajakulasingam *et al.*, 1998; Ying *et al.*, 1998). However, the functional significance of IgE receptor expression by eosinophils remains a controversial issue.

The low expression levels and the localization of IgE receptors within eosinophils may be underlying this controversy. Emerging evidence suggests that although human eosinophils express FcεRs, these receptors are predominantly localized intracellularly and only a small fraction of the FcεR pool is present at the cell surface (Kayaba *et al.*, 2001; Kita *et al.*, 1999; Smith *et al.*, 2000). In addition, the expression levels of FcεRs on human eosinophils are dependent on the concentration of IgE in the microenvironment (Kayaba *et al.*, 2001; Kita *et al.*, 1999). Hence, the intracellular pool of FcεRs might be required for a rapid cellular response by eosinophils to immunological challenges. Although the surface expression is very low, these receptors can trigger the release of cytokines, induce antibody-dependent cellular cytotoxicity towards helminthes and stimulate eosinophil degranulation (Dombrowicz *et al.*, 2000; Gounni *et al.*, 1994; Kayaba *et al.*, 2001; Khalife *et al.*, 1986; Khalife *et al.*, 1985; Tomassini *et al.*, 1991).

The source of eosinophils might be another critical determinant in certain experimental models. Firstly, there exists a discrepancy in FcεR expression by eosinophils from various species. While both human and rat eosinophils express functional FcεR, mouse eosinophils do not express, at the messenger (m) RNA (ribonucleic acid) nor the protein level, FcεRs (de Andres *et al.*, 1997; Dombrowicz *et al.*, 2000). Furthermore, since the expression of FcεRs on the cellular surface is dependent on IgE concentrations (Kayaba *et al.*, 2001; Kita *et al.*, 1999), differences in IgE levels from patient to patient adds another degree of complexity. Finally, investigators have shown

that hypodense eosinophils but not normodense eosinophils appear to respond to IgE stimulation (Khalife *et al.*, 1986).

In general, the data implicate IgE as a mediator of eosinophil degranulation. Khalife and colleagues showed that cytophyllic IgE induces EPO release specifically in hypodense eosinophils from filariasis-infected patients which correlates with other studies that also failed to observe an IgE mediated degranulation in normodense eosinophils (Abu-Ghazaleh *et al.*, 1989; Khalife *et al.*, 1986). In addition, eosinophils isolated from allergic patients also released EPO in response to IgE stimulation (Tomassini *et al.*, 1991). However, the specific contribution of IgE stimulation to eosinophil activation remains unclear. Mast cells and basophils are more responsive to IgE than eosinophils (Kita *et al.*, 1999). Furthermore, eosinophils released less EDN in response to IgE in comparison to IgG (Kaneko *et al.*, 1995b; Kita *et al.*, 1999). In light of current knowledge, additional studies are required to characterize the functional heterogeneity within eosinophil populations and identify physiological and pathological circumstances when IgE plays an active role in stimulating eosinophil effector functions. In addition, the effect of IgE on other cellular functions of eosinophils should be investigated. Notably, IgE can enhance antigen internalization and presentation to T cells by APCs (Maurer *et al.*, 1995; Maurer *et al.*, 1996; Mudde *et al.*, 1995). Thus, the primary role of FcεR on eosinophils may not be to induce cellular activation but rather to promote its function as an APC.

1.6.3.3 Role of Antigen

Immunoglobulin-induced degranulation of human eosinophils may be dependent on antigen specificity. In certain experimental systems, binding to immunoglobulins was insufficient to induce eosinophil degranulation and the presence of the antigen was necessary (Khalife *et al.*, 1985). As a consequence, only low levels of granule proteins were released by eosinophils when stimulated with antibody alone, antigen alone, an irrelevant antigen and an antibody recognizing an unrelated antigen (Khalife *et al.*, 1985). Furthermore, cytophilic immunoglobulins present on the surface of circulating and tissue-dwelling eosinophils stimulate degranulation only upon ligation with the antigen (Khalife *et al.*, 1986; Tomassini *et al.*, 1991). Hence, a mechanism regulating the release of granule proteins by eosinophils at sites of inflammation exists and prevents the premature degranulation of circulating eosinophils as the cells encounter immunoglobulins within blood. Although eosinophil degranulation was induced in other experimental models by immunoglobulins in the absence of antigen, this cellular response occurs only when the stimulus is immobilized to a surface (Kaneko *et al.*,

1995a). Under these circumstances, the non-phagocytosable surfaces may serve only as a substitute for the physiological antigen. Therefore, the antigen may activate eosinophil degranulation via 2 mechanisms: 1) the binding of the antibody to the antigen may cause a conformational change in the structure of the immunoglobulin permitting the transduction of signals via the Fc receptor and, 2) the antigen serves as a surface for cellular adhesion, a necessary co-stimulatory signal for eosinophil activation.

1.6.4 Lipid mediators and Steroids

During an inflammatory response, numerous lipid mediators are secreted by effector cells. These molecules are principally the products of arachidonic acid metabolism which include prostaglandins, leukotrienes and lipoxins. Other important bioactive lipids are PAF and steroids. Importantly, the lipids have been directly implicated in the pathogenesis of allergic disorders (Chung and Barnes, 1991; Drazen, 1998; Page, 1991). Lipids may potentially affect disease processes by activating eosinophil effector functions.

1.6.4.1 Mast cell-derived factors

Mast cells are key effector cells in the early-phase response during allergic diseases and may secrete bioactive lipids to modulate the leukocyte subsets implicated in the late-phase response. Mast cells secrete prostaglandins, leukotrienes and histamine upon cross-linking of FcεRI with IgE-antigen complexes (Metcalfe *et al.*, 1997). When tested for the ability of various bioactive lipids to activate human eosinophils, only LTB4 and PAF induced the release of ECP (Kroegel *et al.*, 1988; Kroegel *et al.*, 1989; Soyombo *et al.*, 1994; Takafuji *et al.*, 1998). Furthermore, PAF has been shown to be one of the most potent stimuli of eosinophil degranulation. β2-integrin engagement is also essential for PAF-mediated eosinophil degranulation (Horie and Kita, 1994). Interestingly, prostanoids which signal through the DP and EP receptors inhibited the release of ECP by eosinophils (Butchers and Vardey, 1990).

1.6.4.2 Priming by eicosanoids

Other bioactive lipids prime eosinophil degranulation. Indeed, certain eicosanoids enhance the degranulation of human eosinophils in response to various secretory stimuli. Although 5-oxo-eicosatetraenoate (ETE) induces a weak degranulation response within eosinophils, it could enhance the secretory signals elicited by PAF, C5a and LTB4 (O'Flaherty *et al.*, 1996). Derivatives of 5-oxo-ETE such as 5-oxo-15-hydroxy-ETE and 5-hydroxy-ETE provided a milder response while other eicosanoids

were inactive under all conditions (O'Flaherty *et al.*, 1996).

1.6.4.3 Sex hormones

A link between the prevalence of asthma during pregnancy suggested a role for sex hormones in regulating eosinophil function. Individually the hormones β-estradiol, progesterone and testosterone had no effect on eosinophil degranulation (Hamano *et al.*, 1998). However, the combination of β-estradiol and progesterone induced eosinophil effector functions (Hamano *et al.*, 1998). Unfortunately, the study did not investigate a possible role for the sex hormones as priming agents. Adhesion processes play a crucial role in the regulation of eosinophil activation. Since the sex hormones modulate the adhesive properties of eosinophils (Hamano *et al.*, 1998), it is possible that sex hormones may enhance or inhibit eosinophil degranulation stimulated by a strong secretagogue.

1.6.5 Complement fragments

Complement proteins play an obscure role during immunological responses. Several receptors for complement factors are present on eosinophils. Engagement of these receptors stimulated eosinophil degranulation. Serum-coated beads induced the release of ECP from human eosinophils (Carlson *et al.*, 1993; Winqvist *et al.*, 1984). Furthermore, purified complement fragments C3a, C3b and C5a all stimulated the release of granule proteins (Carlson *et al.*, 1993; Takafuji *et al.*, 1996; Takafuji *et al.*, 1994). Eosinophil degranulation stimulated by serum, C3a and C5a could be enhanced by IL-3 and IL-5 (Carlson *et al.*, 1993; Takafuji *et al.*, 1996). In conclusion, complement proteins may potentiate eosinophil degranulation during an inflammatory response.

1.6.6 Eosinophil cytotoxic granule proteins

Although the cationic granule proteins possess cytotoxic properties, specific molecules have been shown to regulate secretory pathways of mast cells, neutrophils, basophils and platelets through a non-cytolytic mechanism (Moy *et al.*, 1990; O'Donnell *et al.*, 1983; Rohrbach *et al.*, 1990; Zheutlin *et al.*, 1984). Both MBP and EPO, but not ECP, can also stimulate eosinophil degranulation independently of their cytolytic properties (Kita *et al.*, 1995). Importantly, the response of human eosinophils to EPO and MBP is a dose-, temperature- and calcium-dependent response and could be inhibited by various pharmacological agents (Kita *et al.*, 1995). Therefore, an autocrine mechanism exists to maintain activation signals upon the stimulation of eosinophil degranulation by exogenous agonists.

1.6.7 Adhesion molecules

Adhesion molecules regulate the selective recruitment of leukocytes to sites of inflammation in allergic diseases. Notably, tissue eosinophilia-associated with experimental asthma was abolished by inhibitory antibodies targeted against integrins expressed by eosinophils and in mice-deficient for ligands of these integrins (Broide *et al.*, 1998; Nakajima *et al.*, 1994). In addition, integrin-mediated cellular adhesion may also play an important role in modulating human eosinophil activation in response to secretagogues. Evidence supporting this notion include kinetic studies depicting that cellular adhesion precedes eosinophil degranulation, soluble stimuli fail to induce eosinophil degranulation whereas immobilized ligands activate the release of EDN and inhibition of cellular adhesion by stirring the cell culture prevents PAF and GM-CSF stimulation of eosinophil degranulation (Horie and Kita, 1994; Kaneko *et al.*, 1995a).

1.6.7.1 Mac-1

Integrins are heterodimers composed of an α- and a β-subunit. Eosinophils express several members of the β_1 and β_2 integrin families including very-late antigen-4 (VLA-4; α_4/β_1), lymphocyte function-associated antigen-1 (LFA-1; α_L/β_2) and Mac-1 (α_M/β_2). The expression of the adhesion molecules is modulated by the activation status of eosinophils (Li *et al.*, 1996). Notably, Mac-1 is up-regulated in eosinophils from allergic patients and further increased upon migration to sites of allergen provocation (Busse *et al.*, 1996). Correlation of the expression profile of Mac-1 and eosinophil activation suggests a potential role for this integrin in modulating eosinophil effector functions. Indeed, antibodies targeted against the α_M and β_2 subunits but not antibodies recognizing the α_L subunit of LFA-1 inhibited human eosinophil degranulation stimulated by a range of physiological stimuli (Horie and Kita, 1994; Kaneko *et al.*, 1995a). Moreover, direct ligation of Mac-1 induced intracellular signaling events culminating in the release of EDN (Kato *et al.*, 1998a; Kato *et al.*, 1998b).

Mac-1 may interact with a number of ligands including iC3b, fibrinogen, intercellular adhesion molecule (ICAM)-1 and ICAM-2. Several molecules may modulate β_2-integrin dependent eosinophil degranulation. Although human eosinophils do not spontaneously bind to ICAM-1, GM-CSF induced cellular adhesion to this substrate which led to activation of eosinophil effector functions including degranulation (Nagata *et al.*, 1998). Furthermore, a report depicted the potential of soluble ICAM-1 to induce human eosinophil secretion of granule contents (Chihara *et al.*, 1995). However, ICAM-1 may not serve as the primary ligand *in vivo*. Degranulation of human eosinophils co-cultured with respiratory syncytial virus-infected pulmonary epithelial

cells was a β_2-integrin dependent but ICAM-1 independent process despite ICAM-1 expression being up-regulated in virus-infected epithelial cells (Olszewska-Pazdrak *et al.*, 1998). Components of the extracellular matrix may also modulate Mac-1 activation of eosinophils. Fibrinogen synergistically activated human eosinophil degranulation in the presence of IgG tethered to beads (Kaneko *et al.*, 1995a). Hence, both extracellular matrix proteins or cell-bound ligands may serve as a counterpart for Mac-1 *in vivo*.

1.6.7.2 ICAM-1

Although ICAM-1 may modulate eosinophil degranulation through its interaction with Mac-1, it may also directly affect intracellular signaling pathways linked to eosinophil degranulation. ICAM-1 is absent in peripheral blood eosinophils but its expression is induced upon migration of eosinophils to sites of allergic disease (Hansel *et al.*, 1991a). The expression of ICAM-1 on the surface of human eosinophils can be up-regulated *in vitro* by GM-CSF and TNF-α leading to a subsequent release of granule proteins (Horie *et al.*, 1997a). Treatment with a blocking antibody raised against ICAM-1 significantly inhibited eosinophil degranulation (Horie *et al.*, 1997a).

1.6.7.3 VLA-4/VCAM-1

Eosinophils express VLA-4 which through its interaction with vascular cell adhesion molecule (VCAM)-1 on endothelial cells regulates the recruitment of eosinophils to sites of allergic inflammation (Nakajima *et al.*, 1994). This adhesion system plays a selective role in eosinophil-driven diseases since neutrophils do not express VLA-4. Unfortunately, the evidence linking both adhesion molecules to eosinophil activation is controversial and requires additional experiments to delineate the accurate effects of VLA-4 and VCAM-1. In two independent reports, Nagata and colleagues (Nagata *et al.*, 1995; Nagata *et al.*, 1998) demonstrated that adhesion of human eosinophils to VCAM-1 coated wells induced superoxide production. Furthermore, blocking antibodies to both β_1 and β_2 integrins inhibited the respiratory burst. GM-CSF enhanced the effect of VCAM-1 mediated eosinophil adhesion on the production of reactive oxygen species (Nagata *et al.*, 1998). However, this enhancement was inhibited only by blocking antibodies against β_2 integrins (Nagata *et al.*, 1998). With regards to degranulation, VCAM-1 required GM-CSF as a co-stimulatory molecule to induce the secretion of EDN by human eosinophils (Nagata *et al.*, 1998). Once again, this effect was only inhibited by blocking antibodies against β_2 integrins. How signaling pathways downstream of β_2 integrins cooperate with the VLA-4/VCAM-1 complex in adherent eosinophils remains unclear.

1.6.7.4 Extracellular matrix

Eosinophils adhere to fibronectin in a VLA-4 dependent mechanism. However, divergent experimental conditions have led to contradictory results as to the effect of this adhesion event on eosinophil activation. In an initial study, Neeley and colleagues (Neeley *et al.*, 1994) demonstrated that VLA-4-mediated adhesion to fibronectin primed human eosinophils to degranulate in response to a combination of N-formyl-methionyl-leucyl-phenylalanine (fMLP) and cytochalasin B. Unfortunately, the effects of the pharmacological agent cytochalasin B are ill-defined and may modify the behavior of cells. Other investigators have demonstrated that PAF, IL-5 and C5a but not sIgA-induced EDN release was lower in fibronectin, laminin and collagen, compared to human serum albumin, coated wells (Kita *et al.*, 1996). However, eosinophil degranulation was not affected by coating wells with fibrinogen in lieu of human serum albumin (Kita *et al.*, 1996). The inhibitory effects of fibronectin, laminin and collagen are associated with their ability to bind β_1 integrins. An equilibrium between β_1 and β_2 engagement may be a key regulatory mechanism for eosinophil degranulation.

In conclusion, eosinophil degranulation requires cellular adhesion via β_2 integrins but the role of β_1 integrins in regulating eosinophil activation remains elusive. Additional experiments aimed at understanding the cross-talk between different adhesion systems is necessary to delineate the mechanisms by which adhesion molecules modulate eosinophil effector functions.

1.6.8 Intracellular signaling pathways

The intracellular signaling pathways which regulate eosinophil degranulation are poorly defined. In general, only common second messengers and the protein families implicated in eosinophil activation have been described for certain agonists. However, the specific protein family members remain largely unknown. This review will focus on the signal transduction pathways modulating Fc receptor activation by cytokine priming and immunoglobulin-induced degranulation of human eosinophils (Fig.1.4).

1.6.8.1 Cytokine priming: IL-4 and IL-5 signaling pathways

A potential priming mechanism of eosinophil degranulation is the regulation of the affinity or avidity of immunoglobulin receptors by Th2 cytokines. IL-4, IL-5 and GM-CSF

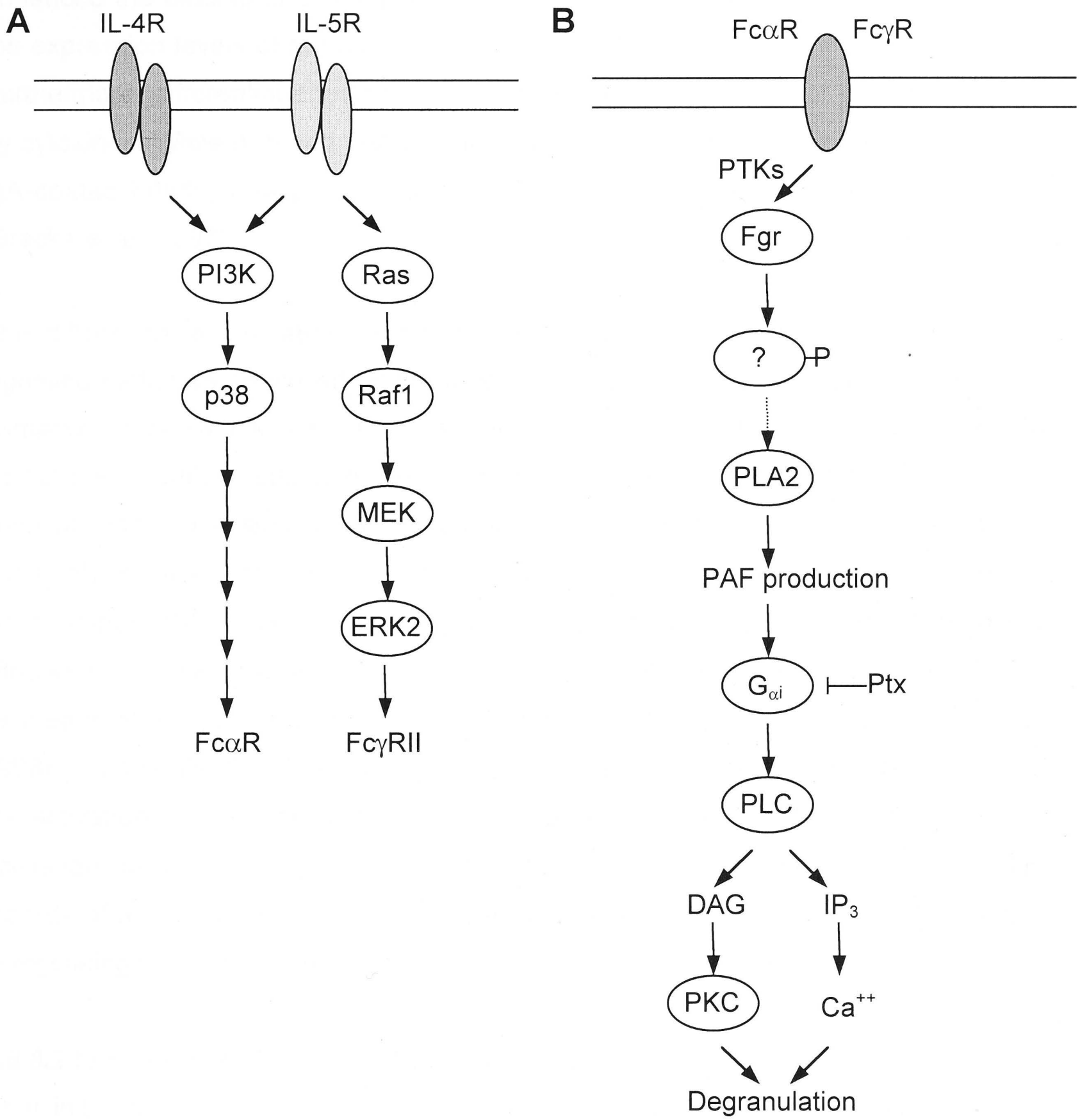


FIGURE 1.4- Intracellular signalling pathways regulating human eosinophil activation. *A.* Differential downstream effectors from the IL-4R and IL-5R result in the activation of specific immunoglobulin receptors in primed eosinophils. *B.* Secretagogues such as immunoglobulins activate a signalling pathway which induces the production and secretion of PAF by eosinophils. The stimulation of a PAF receptor further propagates the signal via classical effectors of G-protein-coupled receptors prompting the eosinophil to undergo degranulation.

enhanced the binding of eosinophils to immunoglobulin-coated beads without altering the expression levels of the receptors (Bracke *et al.*, 1997; Koenderman *et al.*, 1993). Furthermore, differential effects were observed for the regulation of FcγRII and FcαR by cytokines. While IL-5 and GM-CSF increased the binding of eosinophils to IgG and IgA-coated beads, IL-4 primed eosinophils for an interaction with IgA and not IgG (Bracke *et al.*, 1997).

The difference in activation properties of IL-4 and IL-5 relies on the intracellular signaling pathways employed by the receptors (Fig.1.4*A*). Although cytokine receptors primarily signal via Jak-Stat pathways, current evidence suggests that these pathways do not play a critical regulatory role in eosinophil activation but other cellular functions such as eosinophil survival (Pazdrak *et al.*, 1998). However, both IL-4 and IL-5 positively regulate the function of FcαR by signaling via a pathway involving phosphoinositol-3-kinase (PI3K), which directs the activation of p38 MAP kinase (Bracke *et al.*, 1998; Coffer *et al.*, 1998). In contrast, the activation of FcγRII is affected by means of the p21ras-Raf1-MEK-ERK2 pathway (Bracke *et al.*, 1998; Coffer *et al.*, 1998). Importantly, the Raf-1 kinase and its downstream effectors may be required for the activation of eosinophils, in general, since this pathway did not only affect FcγRII activation but also Mac-1 expression and degranulation (Pazdrak *et al.*, 1998). The inability of IL-4 to activate the latter signaling cascade explains its lack of involvement in regulating FcγRII function (Bracke *et al.*, 1998).

1.6.8.2 Immunoglobulin induced eosinophil degranulation

After initial priming events, human eosinophils will release optimal levels of granule proteins when stimulated by a secretagogue. Immunoglobulins are potent secretory signals for eosinophils. In particular, IgG and IgA induce eosinophil degranulation via the FcγRII and FcαR, respectively (Abu-Ghazaleh *et al.*, 1989; Kaneko *et al.*, 1995b; Motegi and Kita, 1998). Recent reports have provided additional insight into the molecular events downstream of these receptor systems (Fig.1.4*B*).

Activation of Fc receptors leads to a rapid induction of protein-tyrosine phosphorylation with a subset of these proteins potentially phosphorylated by the protein-tyrosine kinase Fgr (Kato *et al.*, 1995; Kato *et al.*, 1997; Kita *et al.*, 1994). Although FcR are not G-protein coupled receptors, pertussis toxin, an inhibitor of the $G_{\alpha i}$ subunit of heterotrimeric proteins, inhibited eosinophil degranulation (Kita *et al.*, 1991b; Kita *et al.*, 1994). This observation led investigators to hypothesize and demonstrate that the signal is subsequently transduced to PLA2, which induces the synthesis of PAF

(Bartemes *et al.*, 1999). The endogenous production of PAF is essential for eosinophil degranulation. Eosinophils express two receptors for PAF which are seven transmembrane domain, G-protein coupled receptors. However, it is unknown whether PAF must be secreted to subsequently activate a receptor on the cell surface or whether PAF can directly activate an intracellular receptor molecule. The activation of a PAF receptor couples the heterotrimeric protein to downstream effectors, including phospholipase C (PLC) (Kita *et al.*, 1991b). The hydrolysis of phosphoinositols catalyzed by PLC results in the production of inositol 1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DAG) which, respectively, activate an intracellular increase in calcium and protein kinase C (PKC). These mediators appear to play important roles in the process of eosinophil degranulation as the addition of pharmacological agents such as calcium ionophores and phorbol esters to eosinophil cultures induces release of granule proteins (Egesten *et al.*, 1993; Fukuda *et al.*, 1985). The regulated exocytosis of granule proteins may require vesicle (v)- soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)s and target (t)-SNAREs, critical effector molecules in the fusion of vesicles with the plasma membrane. Vesicle-associated membrane protein (VAMP)-2 has been identified as an important v-SNARE in the modulation of piecemeal degranulation but the t-SNAREs involved in eosinophil degranulation have yet to be identified (Hoffmann *et al.*, 2001; Lacy *et al.*, 2001). Two potential candidates include synaptosomal-associated protein (SNAP)-23 and syntaxin-4 (Logan *et al.*, 2002). PKC and calcium may relate their signals to v- and t-SNAREs, the terminal effector proteins in the process of exocytosis.

Although the signal transduction pathways regulating eosinophil degranulation are poorly defined, the advent of eosinophil cell lines and novel methodologies for the manipulation of primary eosinophils will lead to additional breakthroughs.

1.6.9 Model of eosinophil degranulation

The signaling networks regulating eosinophil degranulation are complex and remain only partially understood. However, a model of eosinophil degranulation (Fig.1.5) may be developed based on current knowledge.

The mechanisms regulating eosinophil degranulation encompass 5 steps which are required for optimal release of granule proteins: 1) eosinophils are primed by cytokines and/or chemokines; 2) secretagogues such as immunoglobulins, bioactive lipids or complement fragments activate eosinophil secretory pathways; 3) intracellular signaling

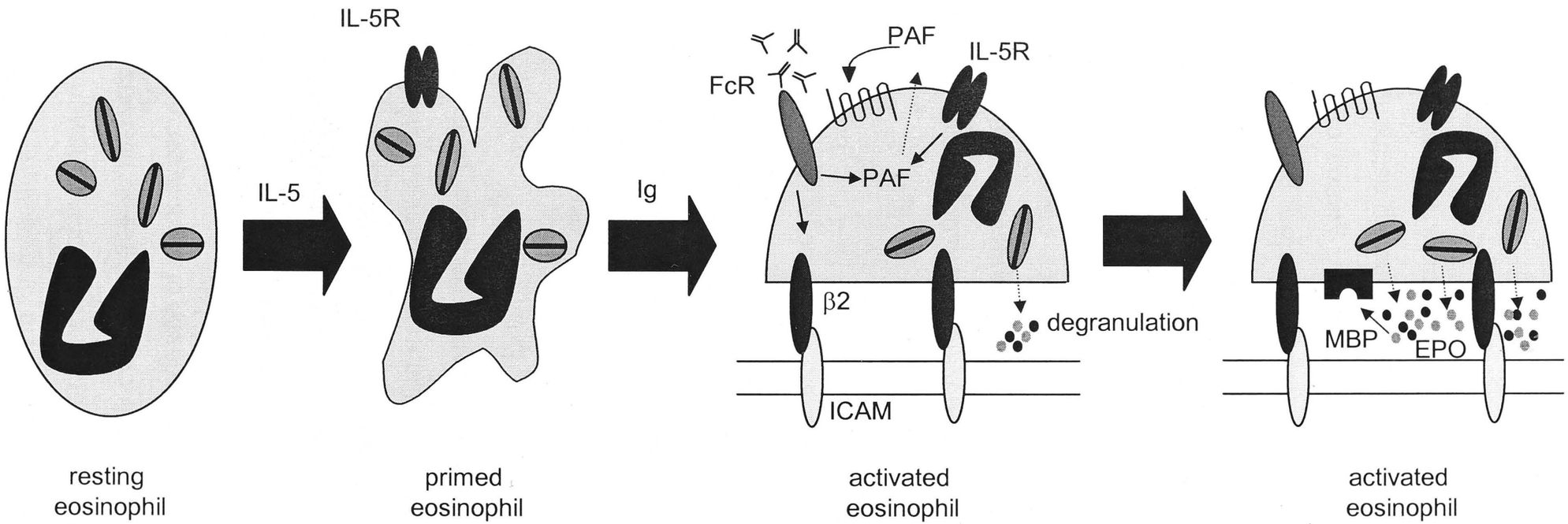


FIGURE 1.5- Model of eosinophil degranulation. Eosinophil degranulation is regulated by several mechanisms which are required for optimal release of granule contents: 1) eosinophils are primed by cytokines (e.g. IL-5) and chemokines; 2) eosinophils are activated by secretagogues such as immunoglobulins; 3) these secretagogues induce the endogenous production of PAF which signals in an autocrine or paracrine manner; 4) adhesion via β_2- integrins is a required co-stimulatory signal; 5) once secreted, MBP and EPO can further stimulate eosinophil degranulation.

cascades downstream of the receptors for secretagogues lead to the endogenous production of PAF which signals either via intracellular receptors or in an autocrine/paracrine manner; 4) the induction of eosinophil adhesion via β2-integrins is an essential co-stimulatory pathway; 5) the cytotoxic cationic proteins released into the extracellular space can further stimulate the release of granule contents.

This paradigm provides a mechanism for the tight control of eosinophil degranulation which is essential due to the cytotoxic properties of eosinophil-derived mediators. Optimal release of granule contents will only occur when all 5 signals are present during the inflammatory response. Unfortunately, eosinophil degranulation may be difficult to arrest once the process is initiated because of the autocrine signals generated by activated eosinophils.

1.6.10 Anti-inflammatory drugs

Numerous drugs have been marketed for their ability to suppress inflammatory responses associated with allergic diseases. The most popular therapeutic agents are the glucocorticoids, β2-adenoreceptor agonists, the non-selective and selective phosphodiesterase inhibitors and H1 blockers. The mechanisms for the effect of these drugs on immune networks is poorly characterized.

The contribution of eosinophils to the development of allergic disease has been appreciated only recently. Inhibitors of eosinophil function would provide a means for relief of symptoms associated with eosinophil-driven diseases. The drugs currently utilized for the treatment of asthma are being tested for a potential direct effect on eosinophil functions including chemotaxis, superoxide production, leukotriene synthesis and degranulation. The majority of drugs have no or little effect on eosinophil degranulation. The glucocorticoids, cetirizine, disodium cromoglycate, denbufylline and ketotifen were ineffective in inhibiting eosinophil secretory processes (Ezeamuzie and Al-Hage, 1998; Kita *et al.*, 1991a; Meng *et al.*, 1997; Okada *et al.*, 1994). However, glucocorticoids may play a role in regulating IL-5 signaling as the IL-5 enhancement of eosinophil degranulation was inhibited by cyclosporin A and rapamycin (Meng *et al.*, 1997).

Certain molecules displayed an intermediate effect on eosinophil activation. Salbutamol and salmeterol were both effective inhibitors of superoxide generation stimulated by PAF (Ezeamuzie and Al-Hage, 1998). However, the inhibition of eosinophil degranulation by salbutamol/ salmeterol was dependent on the

secretagogue. Both drugs reduced the level of EPO release induced by fMLP but had no effect on C5a-induced eosinophil degranulation (Ezeamuzie and Al-Hage, 1998).

The compounds with the greatest potential in regulating eosinophil function include sulochrin, the β2-adenoreceptor agonists formaterol and procaterol, and the phosphodiesterase inhibitors theophylline and isomethylxanthine (Eda *et al.*, 1993; Ezeamuzie and Al-Hage, 1998; Ohashi *et al.*, 1998; Okubo *et al.*, 1997). All molecules were effective inhibitors of eosinophil degranulation induced by the most potent physiological secretagogues including PAF and sIgA. However, numerous studies suggest that the ideal strategy is the use of a combination of drugs which affect different components of the degranulation process. The inhibition of eosinophil degranulation by β2-adenoreceptor agonists was enhanced by the addition of a phosphodiesterase inhibitor (Eda *et al.*, 1993; Okubo *et al.*, 1997).

A number of drugs have demonstrated the potential to inhibit eosinophil effector functions. The development of more potent and specific inhibitors of eosinophil degranulation may be a potential method for reducing the severity of symptoms associated with allergic diseases.

1.7 CONCLUDING REMARKS

Eosinophils are considered key effector cells in the pathogenesis of allergic diseases. One potential mechanism for eosinophils to affect disease processes is through the release of cytotoxic cationic proteins by a process called degranulation. Notably, an increase of eosinophil-derived mediators in allergic tissues often correlates with disease severity (Filley *et al.*, 1982; Fujimoto *et al.*, 1997; Louis *et al.*, 2000). The immune networks regulating eosinophil activation include cytokines, lipid mediators, immunoglobulins, complement proteins and adhesion systems. Since animal models are widely employed to dissect the molecular and cellular mechanisms regulating allergic diseases, these models should reproduce disease processes occurring within human disorders. In limited studies, investigators have failed to detect eosinophil degranulation within the submucosa of allergic lungs of mice questioning the validity of utilizing this specie to model the contribution of eosinophils to the pathogenesis of allergic airways disease (Malm-Erjefalt *et al.*, 2001; Stelts *et al.*, 1998). However, emerging evidence suggests that eosinophils can participate in the regulation of disease processes not solely in the submucosa but also in the airway lumen (Hisada *et al.*, 1999; MacKenzie *et al.*, 2001; Mould *et al.*, 1997; Shi *et al.*, 2000; Tomkinson *et al.*, 2001). Therefore, this thesis describes a comprehensive investigation in the activation

status of eosinophils in a mouse model of allergic airways disease including potential reasons for the discrepancy in eosinophil degranulation within the lung submucosa of allergic mice and asthmatics. Animal models of experimental asthma may provide a tool to further elucidate the mechanisms regulating eosinophil activation *in vivo*. A better understanding of mechanisms modulating eosinophil degranulation may provide a platform for the development of novel therapeutic compounds for the treatment of allergic inflammatory diseases.

Specific aims of this thesis-

1)To determine whether eosinophils degranulate to contribute to the pathogenesis of disease in a mouse model of allergic airways inflammation.

2)To compare the mechanism of degranulation between human and mouse eosinophils.

3)To develop a method for the purification of eosinophils from the bone marrow, blood and airway lumen of allergic mice.

2

Eosinophil degranulation in the allergic lung of mice primarily occurs in the airway lumen

2.1 INTRODUCTION

Eosinophils are regarded as key effector cells in the pathogenesis of allergic diseases (Rothenberg, 1998; Wardlaw *et al.*, 1995). These leukocytes may induce disease through the release of a range of pro-inflammatory molecules and cytotoxic proteins including MBP, EPO, and eosinophil ribonucleases from their granules. Notably, MBP-1 has been associated with the induction of epithelial damage, edema and AHR to inhaled spasmogens (Frigas *et al.*, 1980; Gundel *et al.*, 1991; Hisamatsu *et al.*, 1990; Uchida *et al.*, 1993). Accordingly, the increased presence of eosinophils and their secreted products in the asthmatic lung often correlates with disease severity and exacerbation of disease (Filley *et al.*, 1982; Fujimoto *et al.*, 1997; Louis *et al.*, 2000).

Cationic cytotoxic proteins are stored in the secondary granules of eosinophils and their release, termed degranulation, may proceed via three mechanisms: i) classical exocytosis, whereby secondary granules directly fuse with the plasma membrane of the cell to release their entire contents into the extracellular environment (Lindau *et al.*, 1993; Nusse *et al.*, 1990); ii) piecemeal degranulation, whereby small vesicles bud from the secondary granules and subsequently transport a subset of the granule proteins to the cell surface, resulting in the progressive loss of secondary granule constituents (Dvorak *et al.*, 1992; Dvorak *et al.*, 1991); and iii) cytolysis, a highly organized process of cell death where loss of the plasma membrane's integrity leads to the release of cellular contents (Erjefalt *et al.*, 1998). These mechanisms of eosinophil degranulation have been well characterized by transmission electron microscopy and all three processes have been observed in tissues from patients suffering from a range of inflammatory disorders (Dvorak, 1994; Dvorak *et al.*, 1993b; Erjefalt *et al.*, 1998; Erjefalt *et al.*, 1999). Notably, in airway tissues from atopic patients, piecemeal degranulation was predominantly observed, although some eosinophils released granule contents by cytolysis (Erjefalt *et al.*, 1998; Erjefalt *et al.*, 1999).

Recently mouse models of $CD4^+$ Th2 lymphocyte immunity have emerged as central tools to study disease mechanisms that underpin allergic disorders (Foster *et al.*, 1996; Hamelmann *et al.*, 1997; Hogan *et al.*, 2001; Kumar and Foster, 2001). Mouse models of allergic airways disease display several characteristic features of human asthma and have been extensively employed to define eosinophil functions in pathogenesis (Foster *et al.*, 2001; Webb and Foster, 1999; Wills-Karp, 2000). Indeed, in mouse models of experimental asthma, the recruitment of eosinophils to the lung has been directly linked to the development of pathogenic features that are hallmarks of the disorder in humans (Foster *et al.*, 1996; Hamelmann *et al.*, 1999a; Justice *et al.*, 2003; Shen *et al.*, 2003;

Tomkinson *et al.*, 2001). However, attenuation (but not abolition) of eosinophil trafficking to the lung does not always correlate with inhibition of disease processes (Corry *et al.*, 1996; Hessel *et al.*, 1997; Hogan *et al.*, 1998) and eosinophil degranulation in the submucosa of the allergic lung of mice (Malm-Erjefalt *et al.*, 2001; Stelts *et al.*, 1998) (a key feature of human disease (Erjefalt *et al.*, 1998; Erjefalt *et al.*, 1999; Filley *et al.*, 1982)) has not been observed. The failure to observe eosinophil degranulation has questioned the relevance of mouse models of allergic disease to understanding the contribution of eosinophils to allergic disorders (Malm-Erjefalt *et al.*, 2001).

Although eosinophil degranulation is not evident in subepithelial regions of the lungs of allergic mice, MBP and EPO have been detected in cell free extracts taken from the bronchoalveolar lavage fluid (BALF) (Hisada *et al.*, 1999; Mould *et al.*, 2000; Tomkinson *et al.*, 2001). Interestingly, eosinophils residing in the airway lumen of allergic mice have been shown to actively participate in immune processes (MacKenzie *et al.*, 2001; Shi *et al.*, 2000). Collectively, these investigations suggest that eosinophils may selectively receive activation signals in the lumenal compartment. Notably, only limited studies have investigated the activation status of eosinophils in the lungs of allergic mice and these have focused on tissue-dwelling cells and not of those residing in the airway lumen (Hamelmann *et al.*, 1999b; Malm-Erjefalt *et al.*, 2001; Stelts *et al.*, 1998).

In this investigation, we extend our characterization of the role of the eosinophil in the pathogenesis of allergic airways disease in mice by analyzing the ultrastructure and activation status of this cell as it migrates from the bone marrow to the airway lumen during inflammation. We detected cell-free EPO in respiratory secretions (BALF), and showed that eosinophils in the airway lumen are highly activated and release granule contents by piecemeal degranulation (the primary mechanism of degranulation in human asthmatic tissue). Furthermore, we link eosinophil activation (evidenced by piecemeal degranulation and the presence of cell free EPO) in the airway lumen with enhanced airways reactivity to methacholine. We speculate that the differences in the degree and site of eosinophil activation/degranulation between mouse models of asthma and human diseased tissue may arise, in part, because of higher thresholds for the activation of the mouse leukocyte in response to secretagogues and also because of the acute (mouse) versus chronic (human) nature of the inflammatory processes.

2.2 MATERIALS AND METHODS

2.2.1 Mice

Male WT BALB/c, and strain matched IL-5 transgenic (Tg) mice (Ref. Dent *et al.,* 1990; backcrossed to the 12th generation) (6 to 8 weeks old) were supplied by the pathogen-free facility, John Curtin School of Medical Research, Australian National University. Mice were treated in accordance with The Australian National University Animal Welfare Guidelines.

2.2.2 Induction of allergic airways disease

Mice were sensitized by an i.p. injection of ovalbumin (OVA; Grade V, Sigma Chemical Co., St-Louis, MO, USA) on days 0 and 12, at a dose of 50 μg in 1 mg of Alhydrogel (CSL Ltd., Parkville, Australia) in isotonic saline (Foster *et al.*, 1996). Nonsensitized mice received 1 mg of Alhydrogel in isotonic solutions (SAL). On days 24, 26, 28 and 30, all groups of mice were exposed to an aerosol formed from a 1% w/v solution of OVA in saline for 3 periods of 30 min, with a 30 min interval between each exposure. The aerosol was generated by a RapidFlow nebulizer bowl (Allersearch, Melbourne, Australia) (Foster *et al.*, 1996). This regime produced maximal inflammatory cell numbers in the bone marrow, blood and pulmonary tissue (Ref. Foster *et al.*, 1996; data not shown). Cellular responses were characterized 16 h after the last aerosol exposure.

2.2.3 Characterization of pulmonary inflammation

Leukocytes recruited to the lungs of control and allergic mice were characterized in the BALF (Foster *et al.*, 1996). Briefly, to lavage the lungs, the trachea was exposed, canulated with a blunt 18-gauge needle, and the lungs were washed twice with 1 ml of PBS. BALF was recovered and centrifuged for 5 min at 300 x g. The supernatant was stored at -70°C for assessment of EPO content. Leukocyte populations in BALF were identified according to standard morphology and quantified as previously described (Foster *et al.*, 1996). Importantly, viability of leukocytes was always very high (>95% according to trypan blue assay).

2.2.4 Collection and processing of samples for transmission electron microscopy

Lung tissue and cells isolated from bone marrow, blood and BALF were processed for transmission electron microscopy. Bone marrow, blood and BALF cells and lung tissue

were isolated (six mice per group) by standard techniques (Foster *et al.*, 1996; Mould *et al.*, 1997; Mould *et al.*, 2000; Webb *et al.*, 2000). Briefly, individual cell populations were pooled and the red blood cells were lysed using an ammonium chloride buffer (0.16 M NH_4Cl, 0.17 M Tris pH 7.2). Subsequently, the cell populations were washed in PBS containing 1 mM EDTA before being fixed in 2% glutaraldehyde in 0.1 M sodium cocadylate, pH 7.4 at 4°C overnight. Isolated lung tissue (3x3x3 mm) was placed immediately in fixative (as above) and incubated at 4°C overnight. All samples were washed in 0.1 M sodium cocadylate buffer. The samples were postfixed in 1% osmium tetroxide for 90 min, stained *en bloc* in 2% uranyl acetate, dehydrated in graded alcohol solutions and embedded in Spur's resin (ProSciTech, Australia). Sections (1 μm) were stained with Toludine Blue to identify intact regions of pulmonary tissue. Ultra-thin sections (80-85 nm) were cut on a Reichert-Jung Ultracut E ultramicrotome. The samples were stained with lead citrate before analysis using an Hitachi transmission electron microscope (H-7000; Hitachi, Japan).

2.2.5 Quantification of eosinophil degranulation by transmission electron microscopy

Initially, the number of eosinophils that were resting, degranulating or apoptotic were defined in various compartments. Degranulating eosinophils were then further delineated from one another based on the mechanism of granule protein release (exocytosis, piecemeal degranulation and cytolysis). Subsequently, the secondary granules of each eosinophil were counted and classified into four categories (see Fig. 2.1: type I, intact; type II, loss of crystal core; type III, loss matrix; and type IV, loss of both crystal core and matrix). The definitions for all groups are based on the method devised by Erjefalt *et al.* (Erjefalt *et al.*, 1998). The extent of degranulation was quantified by means of a degranulation index calculated using the following formula: Degranulation Index = 100 x (number of activated granules, types II to IV / total granules) (Erjefalt *et al.*, 1998).

2.2.6 Eosinophil purification

Eosinophils from the peritoneal cavity of IL-5 Tg BALB/c mice and from the BALF of allergic WT mice were purified by FACS (fluorescence activated cell sorter; FACStar Plus, Becton Dickinson, San Jose, CA) according to their characteristic forward scatter (FSC) vs. side scatter (SSC) plot and light polarization properties as previously described (Mould *et al.*, 1997). The purity of the enriched population was approximately 95% as determined by differential staining with Giemsa-May-Grünwald. Greater than 98% of the purified eosinophils were deemed viable by trypan blue exclusion.

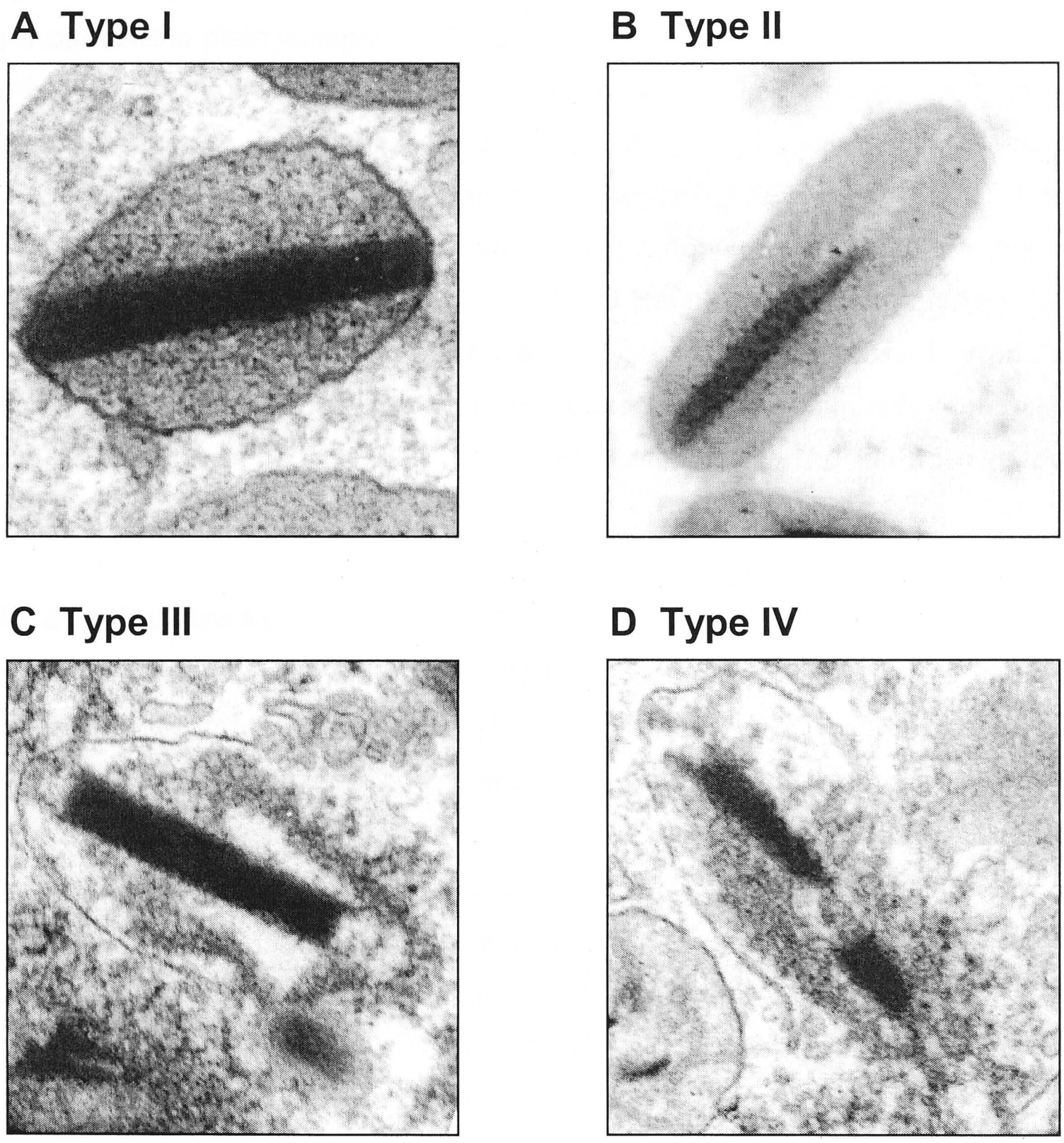


FIGURE 2.1- Phenotypes of secondary granules from eosinophils. *A*, Type I granules are intact granules without loss of the crystal core nor the matrix; *B*, Type II granules have lost proteins associated with the crystal core; *C*, Type III granules display loss within the matrix compartment; *D*, Type IV granules have lost constituents within both the crystal core and the matrix.

2.2.7 Measurement of AHR

Airways reactivity to methacholine was measured in conscious, unrestrained mice using a barometric plethysmograph (Buxco, Troy, NY) as previously described (Webb *et al.*, 2000). Briefly, mice were placed in a plethysmograph chamber and exposed to an aerosol of water to obtain baseline readings of enhanced pause (Penh). Mice were then exposed to cumulative concentrations of methacholine ranging from 3.1 to 50 mg/ml. The aerosol was generated by an ultrasonic nebulizer and drawn through the chamber for 2 min. The inlet was then closed and Penh readings taken for a further 3 min. Data were averaged and reported as the percentage increase in Penh over baseline. Penh values are only shown at the concentration which induced the maximal response but data are reflective of responses obtained with the full dose response curve.

2.2.8 Adhesion Assay

Eosinophil adhesion was monitored in triplicate samples by measuring eosinophil peroxidase content of adherent cells. The cells were cultured in 96-well flat-bottom tissue culture plates, which were coated with 2.5% bovine serum albumin (BSA) in PBS for 2 h at 37°C. The wells were rinsed three times with RPMI-1640. After purification, eosinophils were washed and resuspended in RPMI-1640 at a concentration of 5-7.5x10^5 cells/ml. A 100 µl aliquot of the eosinophil suspension was added to each well. Stimulation of the eosinophils was initiated by the addition of an equal volume of the stimulus dissolved in RPMI-1640. The eosinophils were cultured for various periods of time at 37°C in a humidified atmosphere containing 5% CO_2. After the incubation, each well was gently washed three times with 200 µl of warm PBS to remove non-adherent cells. EPO was extracted by lysing the eosinophils with 200 µl of 0.22% cetyltrimethylammonium bromide (CTAB) dissolved in 10 mM HEPES buffer pH 8.0. Eosinophil adhesion was reported as a percentage of the EPO extracted from adherent cells according to the formula:

% Adhesion = (EPO in adherent fraction / Total EPO added to well) X 100

The denominator was determined by measuring EPO content in an equivalent aliquot of cells added at the start of the experiment. The enzyme was extracted using 0.22% CTAB dissolved in 10 mM HEPES buffer pH 8.0. The samples were frozen at -70°C until EPO content was measured using a colorimetric assay.

2.2.9 *In vitro* degranulation assay

Eosinophils from the peritoneal cavity of IL-5 Tg mice, total BALF cells or purified BALF eosinophils from allergic WT mice were incubated in the presence of various stimuli to induce degranulation. The assay was performed in 24-well flat-bottom tissue culture plates that were coated with either a 2.5% BSA solution or a 1:10 dilution of sera, isolated from control or allergic mice, for 2 h at 37°C. Approximately $5x10^5$ cells were added to each well in a volume of 250 µl of RPMI-1640. An equal volume of culture media, which contained the stimulus, was added. The leukocytes were cultured for 0-4 hours at 37°C in a humidified atmosphere containing 5% CO_2. After incubation, the plates were centrifuged at 300 xg for 5 min. The supernatant was collected and stored at -70°C until assay for MBP.

The morphology of cultured eosinophils was assessed by transmission electron microscopy. The cells were cultured as above, however, an insert, which was coated in succession with poly-L-lysine (10 mg/ml) and then BSA (2.5% w/v in PBS) was introduced into the well. The cells were stimulated for 3 h in the presence or absence of 10^{-7} M phorbol-12-myristate-13-acetate (PMA). After the incubation, the inserts were gently rinsed with warm PBS three times. The cells were fixed in 2% glutaraldehyde dissolved in 0.1 M sodium cacodylate buffer pH 7.4 at 4°C overnight. The subsequent steps were performed as described above.

2.2.10 Detection of EPO and MBP

EPO content in cell-free BALF and eosinophil lysates was measured using a colorometric assay (Bozeman *et al.*, 1990; Schneider and Issekutz, 1996). Briefly, a 75 µl aliquot of sample was transferred to a 96-well microtiter plate and the reaction was initiated by the addition of 75 µl of substrate solution (12 mM *o*-phenylene diamine, 0.005% H_2O_2 in 10 mM HEPES pH 8.0). CTAB is not only a detergent used to disrupt membranes but also serves as a source of bromine ions required by EPO. The final concentration of CTAB in the reaction was 0.11% w/v and was present in the lysates or added to the substrate solution when analyzing BALF. The enzymatic reaction was stopped after a 30 min incubation at room temperature by the addition of 50 µl of 4 N H_2SO_4. Absorbance at 490 nm was measured using a Thermomax microplate reader and data was analyzed using SoftMax (version 2.01; Molecular Devices Corp, Melon Park, CA).

The presence of MBP in supernatants from cultured eosinophils was assessed using an immunodot blot assay, developed with Western Blue as previously described (Mould *et al.*, 2000).

2.2.11 Statistical analysis

Data are presented as the mean ± standard error of the mean (SEM) except for the data describing a frequency of occurrence in a sample population, which were presented as a percentage. Statistical significance of differences between experimental groups was assessed with Student's unpaired *t* test or Chi-Square analysis. Differences in means were considered significant if $p < 0.05$.

2.3 RESULTS

2.3.1 Detection of eosinophil granule proteins in cell-free BALF of allergic mice

The lungs of allergic -WT and -IL-5 Tg mice were characterized by the infiltration of eosinophils (Fig. 2.2*A*), the presence of EPO (Fig. 2.2*B*) in cell-free BALF and the development of AHR in response to methacholine (Fig. 2.2*C*). The eosinophil was the predominant leukocyte present in BALF, representing 70% and 89% of BALF cells recovered from WT and Tg mice, respectively. A significant increase in the numbers of lymphocytes, macrophages and to a lesser degree neutrophils was also observed (results not shown). Notably, elevations in the concentrations of EPO in the BALF directly correlated with the development of both airways eosinophilia and AHR. Eosinophil numbers in the lung of IL-5 Tg mice were greater than that observed in WT mice, however, AHR and EPO levels were similar suggesting that these indices were at maximal levels of detection. These hallmark features of allergic inflammation also correlated with the induction of mucus hypersecretion as previously shown (Webb *et al.*, 2000).

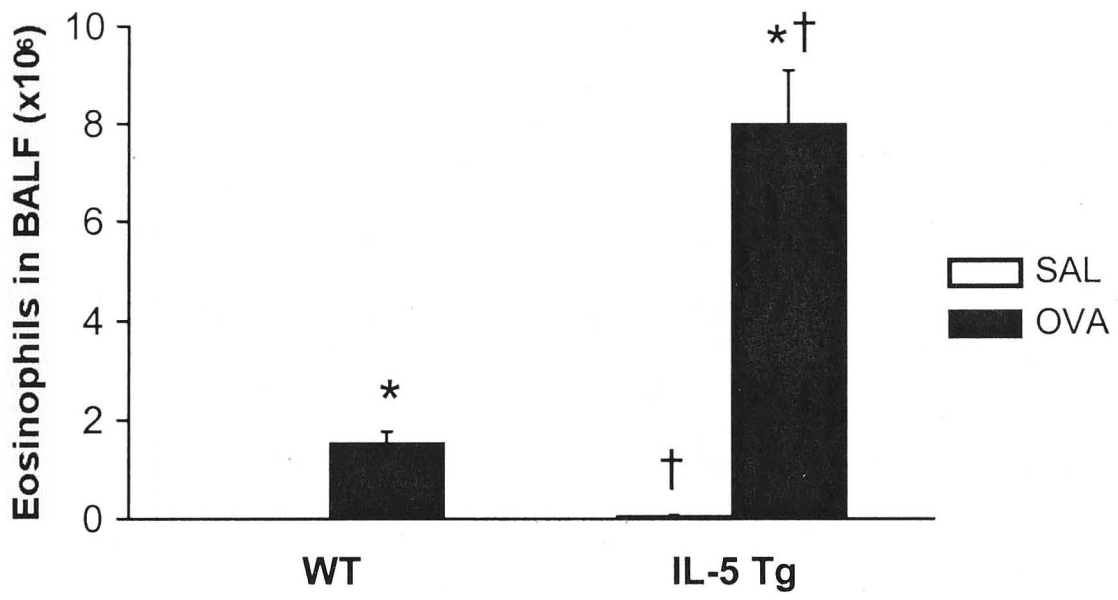
2.3.2 Piecemeal degranulation is the principal mechanism for granule protein release from eosinophils in the allergic lung

Electron microscopic analyses of eosinophils in the bone marrow, blood, airway tissue and BALF were undertaken to determine the extent of cellular activation and the mechanism(s) for granule protein release (Table 2.1 and Fig. 2.3). We observed a subpopulation of activated eosinophils in these four compartments in allergic mice (Table 2.1). Furthermore, all activated eosinophils released granule proteins by piecemeal degranulation. Although some eosinophils from the bone marrow and blood of allergic mice displayed characteristics of piecemeal degranulation, it was normally confined to one or two granules per cell and these granules had lost only a small amount of either the core or matrix (Fig. 2.3*A*). However, once in the lungs, a greater proportion of eosinophils resident in tissues were activated and undergoing piecemeal degranulation (Table 2.1) but the degree of granule protein loss was moderate (Fig. 2.3*B*).

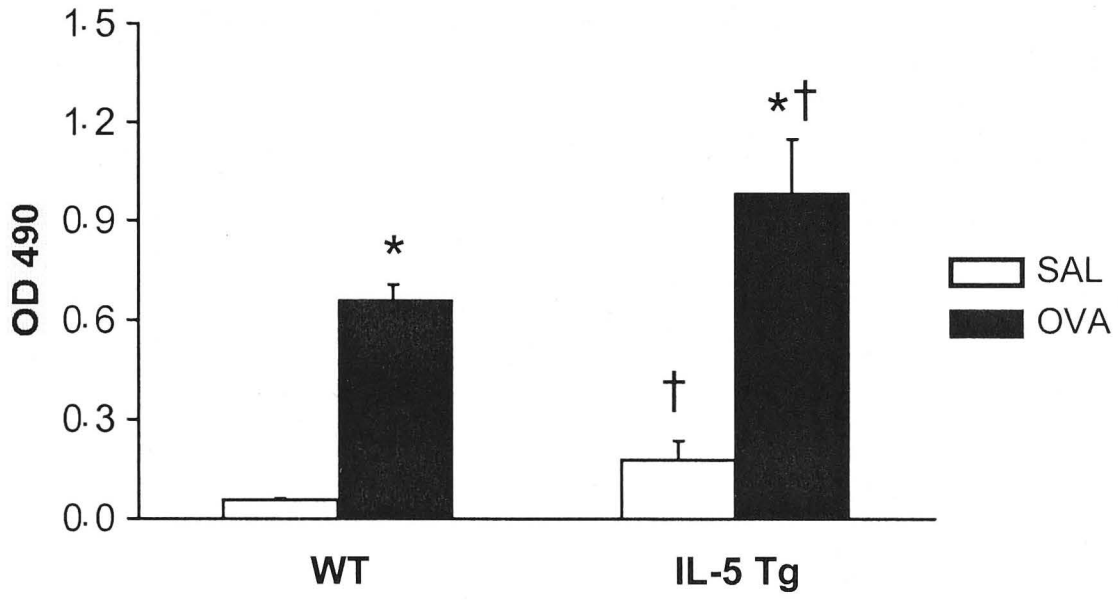
Although increased numbers of activated eosinophils were observed in allergic tissue, the vast majority of cells that were activated and undergoing degranulation were found in the BALF (Table 2.1 and Fig. 2.3). The migration of eosinophils into the airway

FIGURE 2.2- Detection of EPO in cell-free BALF directly correlates with the development of airways eosinophilia and AHR in the allergic lung. OVA sensitization and challenge of WT and IL-5 Tg mice induced hallmark features of allergic disease of the lung. *A*, Number of eosinophils in the airway lumen are reported as the mean total eosinophils isolated within BALF± SEM (*n*= 5-8 mice per group). *B*, EPO concentration in cell-free BALF was determined using a standard colorometric assay. Data were individually collected from 6 mice per group, with each sample analyzed in duplicate. The results are presented as the mean optical density measured at 490 nm ± SEM. *C*, Airways reactivity to methacholine (25mg/ml) was monitored by barometric plethysmography. The data are presented as the percent increase in Penh above baseline values (mean ± SEM, *n*= 6-8 mice per group). * $p < 0.01$ when compared to saline-sensitized/ OVA-challenged group and † $p < 0.05$ when compared to WT mice after identical experimental treatments.

A Eosinophil number



B EPO levels



C Airways reactivity

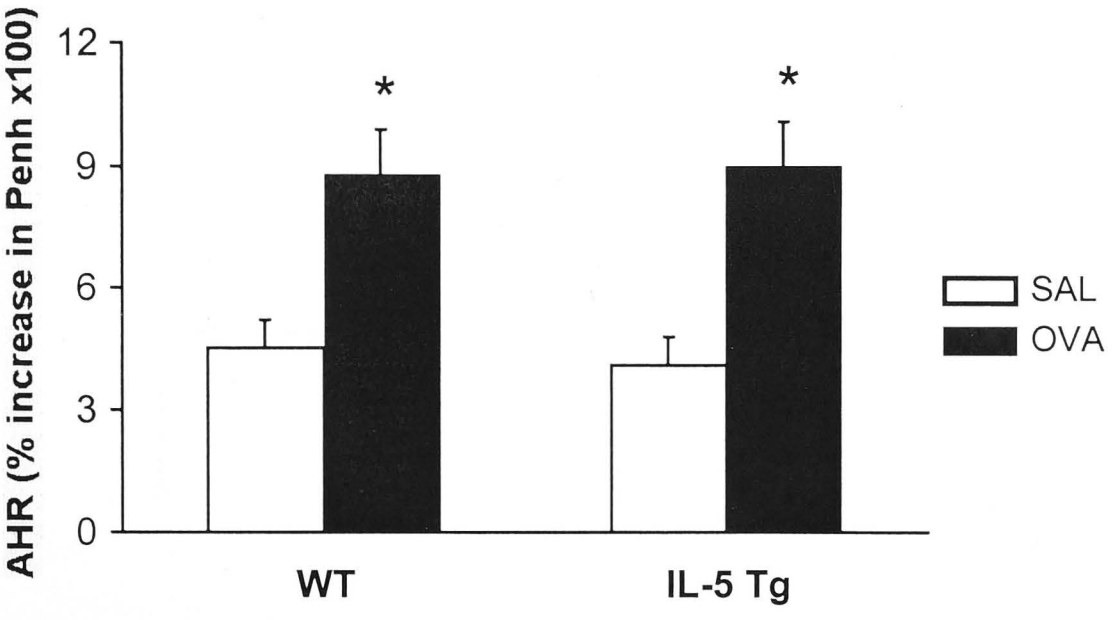


Table 2.1-Eosinophil morphology differs according to tissue compartment occupied.

			% Degranulating			
Genotype	**Eosinophil population**	**% Resting**	**Exocytosis**	**PMD**	**Cytolysis**	**% Apoptotic**
WT	Bone marrow	82	0	18	0	0
	Blood	74	0	24	0	2
	Tissue	64	0	36	0	0
	BALF	33	0	63	0	4
IL-5 Tg	Bone marrow	77	0	23	0	0
	Blood	74	0	26	0	0
	Tissue	60	0	40	0	0
	BALF	17	0	83	0	0

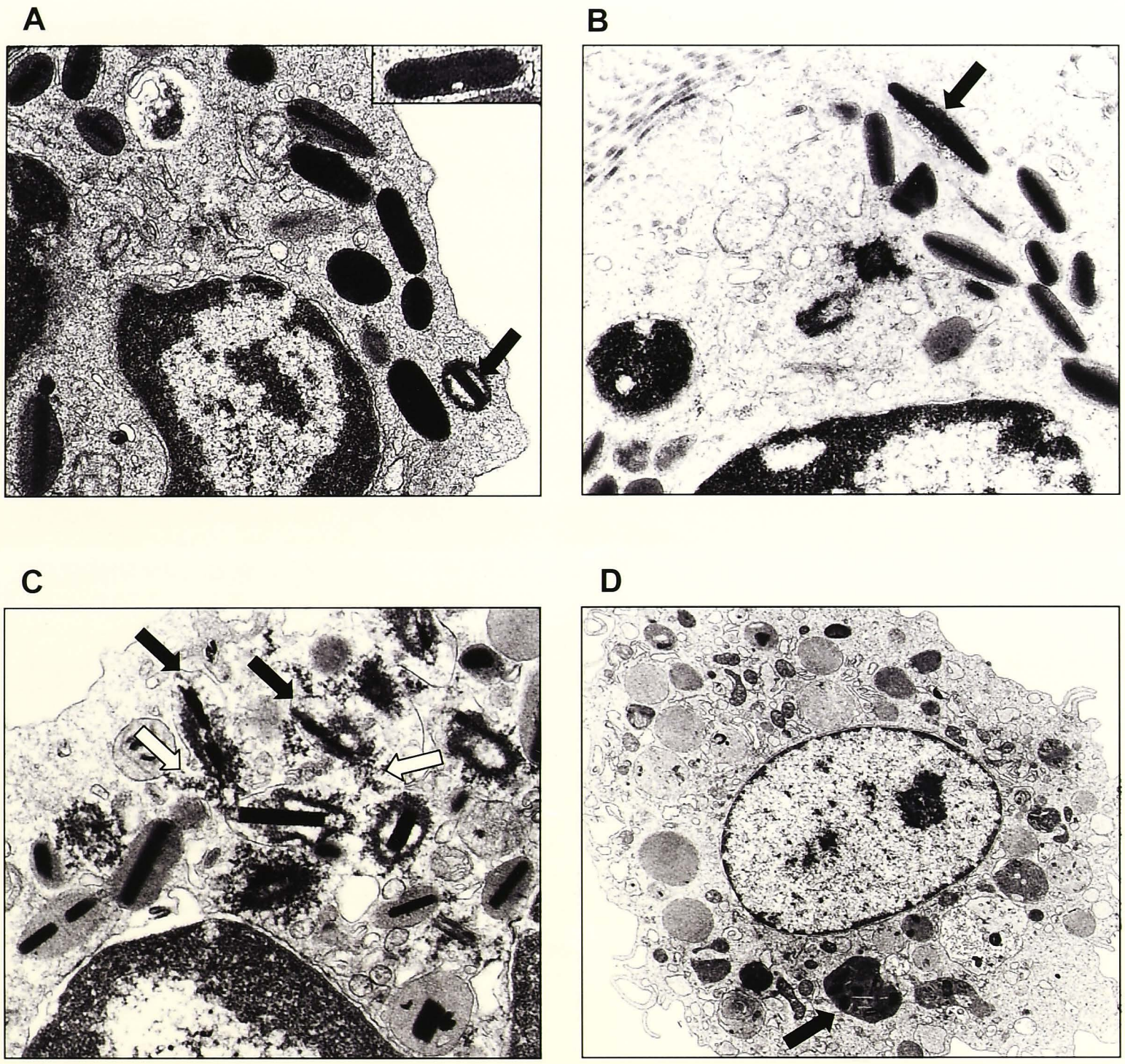


FIGURE 2.3- Electron photomicrographs of mouse eosinophils during allergic airways disease. Eosinophils present in the bone marrow or in the peripheral blood displayed few signs of activation. Only slight changes in the morphology of secondary granules were observed in bone marrow derived eosinophils (arrow and inset in *A*). While a greater proportion of eosinophils in pulmonary tissue were activated (morphological changes in granule structure), the cells in the submucosa released only modest quantities of granule proteins by piece-meal degranulation (arrow depicts type III granule in *B*). However, eosinophils in the airways lumen became highly activated with a concomitant release of granule proteins by piecemeal degranulation. In BALF eosinophils (*C*), a large number of granules were type IV (balck arrow) and were surrounded by small vesicles (white arrow). Macrophages in the airways lumen phagocytosed apoptotic bodies derived from eosinophils (arrow in *D*). Data is representative of 2 samples taken from 6 mice each for bone marrow, blood and airway lumen eosinophils. For tissue-resident eosinophils, lung sections from 4 mice were analyzed.

correlated with a dramatic change in morphology. A large proportion of granules lost either the core, matrix or both granule constituents (Fig. 2.3*C*). Notably, the secondary granules in BALF eosinophils were surrounded by large numbers of small vesicles, which have presumably budded from these granules to facilitate the transport of proteins to the cell surface. These small vesicles were absent in eosinophils from all other compartments. The morphology of BALF eosinophils classically represented piecemeal degranulation.

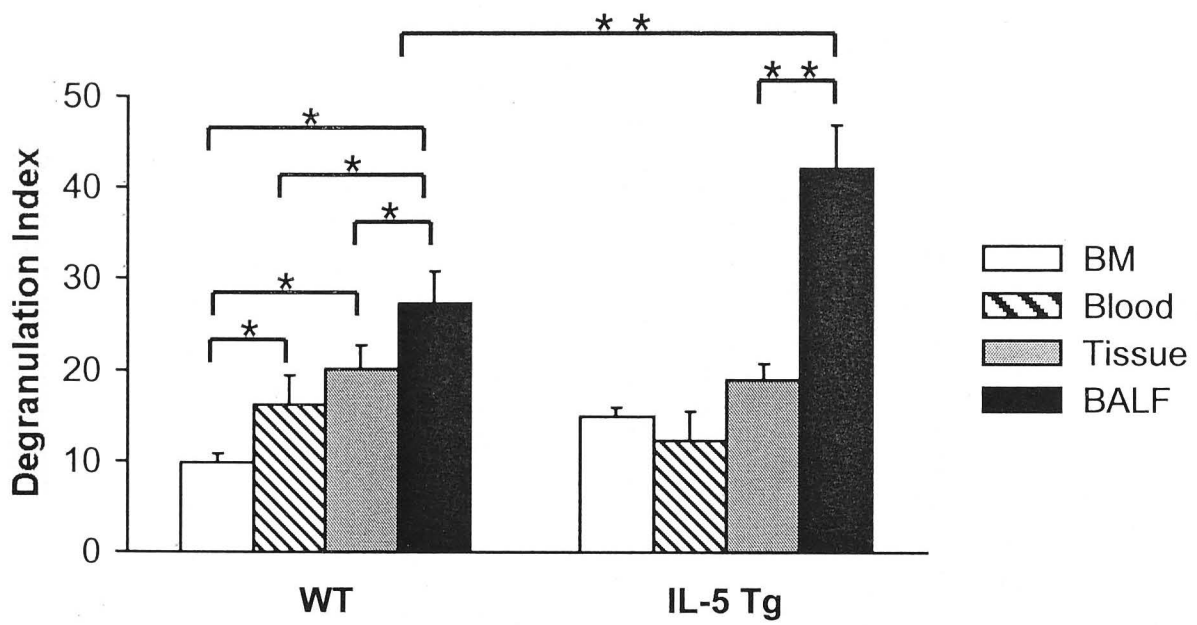
Only a small fraction of eosinophils in the airway lumen of allergic mice were apoptotic (Table 2.1). However, we often observed that macrophages in the airway lumen had engulfed eosinophil constituents including intact secondary granules (Fig. 2.3*D*).

2.3.3 Eosinophils in the airway lumen have the highest activation status

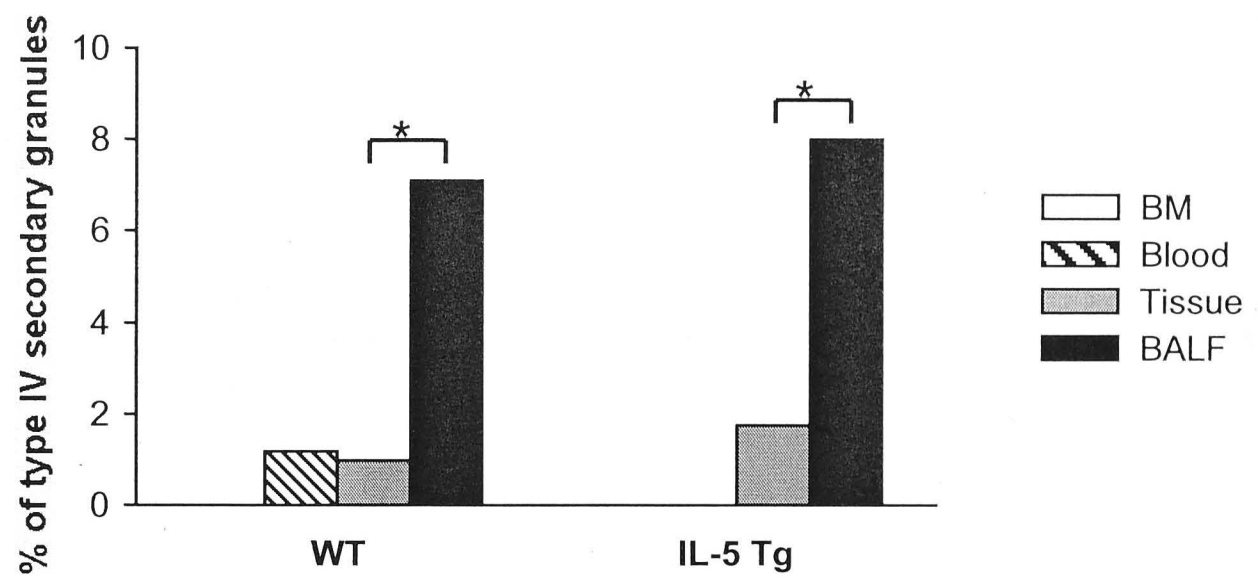
Concurrently with morphological description of eosinophil degranulation, we attempted to quantify their activation status by grading the degree of degranulation per cell in a defined compartment relative to eosinophils isolated from naive (non allergic) mice (Fig. 2.4). Resting eosinophils contained only type I granules, while activated eosinophils possessed both intact granules and those displaying at least a partial loss of granule content (types II-IV). When the data was transformed into the degranulation index (on a scale of 0 to 100, where 0 indicates a resting eosinophil and 100 a fully activated cell with no type I granules) distinct differences in the degree of activation were observed between eosinophils residing in the various compartments (Fig. 2.4*A*). Eosinophils isolated from naive mice had a degranulation index equal to bone-marrow derived eosinophils from allergic mice. Furthermore, in the case of WT mice, the degranulation index of circulating eosinophils increased significantly after allergen provocation (9.9 ± 1.7 vs. 16.2 ± 2.6, $p<0.05$). Although eosinophils in lung tissue of allergic mice were activated (in contrast to control and bone marrow-derived cells), the highest activation status was observed in the airways lumen. This is particularly evident after classifying the eosinophils as low, moderately or highly activated (Fig. 2.4*B*) and when the proportion of type IV granules is compared between eosinophil populations in the various compartments (Fig. 2.4*C*). Movement from the tissue to the airway lumen significantly promoted the loss of both the crystal core and matrix from individual granules. These data directly correlate with the morphological assessment of eosinophil activation (Fig. 2.3 and degranulation index). Several differences were observed between WT and IL-5 Tg eosinophils in the various compartments of allergic mice. Notably, although the proportion of activated eosinophils in IL-5 Tg mice was only slightly increased within any one compartment (Table 2.1), those cells that

FIGURE 2.4- Quantification of the activation status of eosinophils in different compartments of allergic mice. *A,* Changes in the degranulation index as eosinophils migrate to sites of allergen provocation. The degranulation index for each eosinophil was calculated using the formula presented in *Materials and Methods*. The data represent the mean degranulation index ± SEM. Significant differences between groups are indicated as $*p < 0.05$ or $**p < 0.001$. *B,* Proportion of eosinophil populations with low, moderate and high activation status. The cells were classified according to the degranulation index into low (0-20), moderately (20-40) or highly (40-100) activated. *C,* Proportion of type IV granules in different eosinophil populations. The secondary granules were classified and counted as previously described (Erjefalt *et al.,* 1998). The data are presented as the percentage of type IV granules. A minimum of 200 granules were counted for each sample. Significant differences ($*p < 0.01$) were determined using the Chi-square analysis.

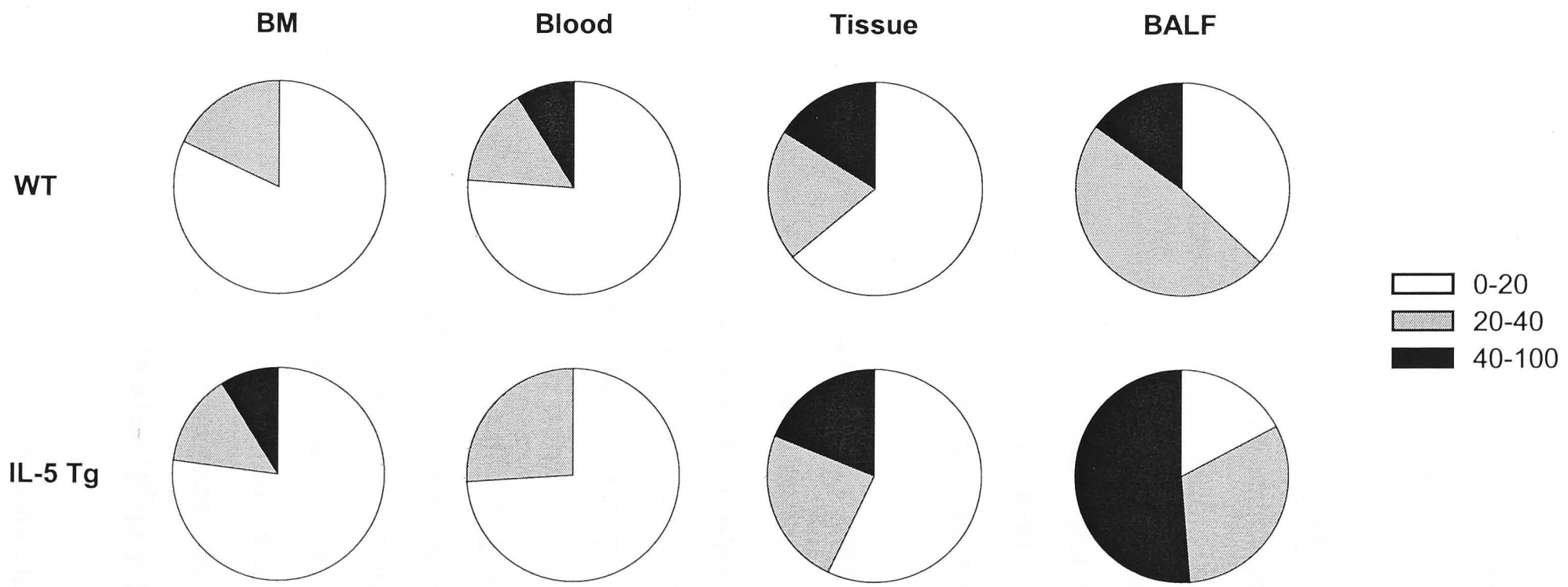
A


Degranulation Index
0
10
20
30
40
50
*
*
*
*
*
**
**
WT
IL-5 Tg
BM
Blood
Tissue
BALF

C


% of type IV secondary granules
0
2
4
6
8
10
*
*
WT
IL-5 Tg
BM
Blood
Tissue
BALF

B


BM
Blood
Tissue
BALF
WT
IL-5 Tg
0-20
20-40
40-100

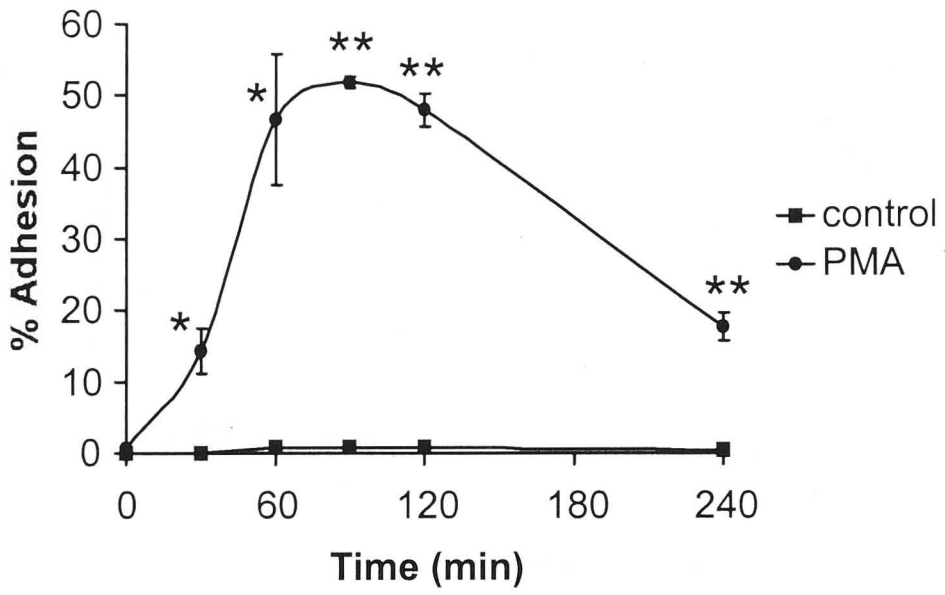
became activated reached higher levels of activation (Fig. 2.4*A,B*). This difference was particularly evident in comparison of BALF populations.

2.3.4 High thresholds for the activation of mouse eosinophils by PMA

Since eosinophil degranulation in the lung of allergic mice was primarily compartmentalized to the airway lumen in contrast to the airways of atopics, we characterized the response of mouse eosinophils to PMA to determine whether significant differences exist in the regulation of human and mouse eosinophil activation. The activation of eosinophils by PMA was both time- and dose-dependent (Fig. 2.5-2.6). The initial response to PMA stimulation was cellular adhesion to the extracellular matrix followed by degranulation (Fig. 2.5). Eosinophil adhesion reached a maximum after approximately 60 to 90 minutes of stimulation, at which time MBP release was first observed. Subsequently, the secretion of MBP increased consistently until the experiment was terminated after 4 hours. Titration of PMA concentration determined that mouse eosinophil activation occurred at concentrations as low as 1 nM but only reached a maximum at 100 nM (Fig. 2.6).

Since high concentrations of PMA were required to induce eosinophil degranulation, we characterized the ultrastructural morphology of the eosinophils to exclude the possibility that cytotoxic effects of PMA were the cause of MBP secretion (Fig. 2.7). Electron microscopic analysis of PMA-stimulated eosinophils confirmed that the cells were highly activated and revealed that piecemeal degranulation was the likely mechanism for MBP release (Fig. 2.7*B*). In unstimulated cells, the secondary granules displayed no alterations as they contained an intact matrix surrounding an electron-dense core (type I) (Fig. 2.7*A*). This core is composed primarily of MBP (Denzler *et al.*, 2000). After stimulation, the release of MBP was accompanied by an increase in the number of secondary granules displaying a complete or partial loss of the core (type II) (Fig. 2.7*B,C*). Accordingly, the degranulation index of eosinophils increased after stimulation with PMA (Fig. 2.7*D*). These results directly correlate with the biochemical analysis of eosinophil activation and indicate that MBP release is not the by-product of cellular degenerative processes.

A Adhesion



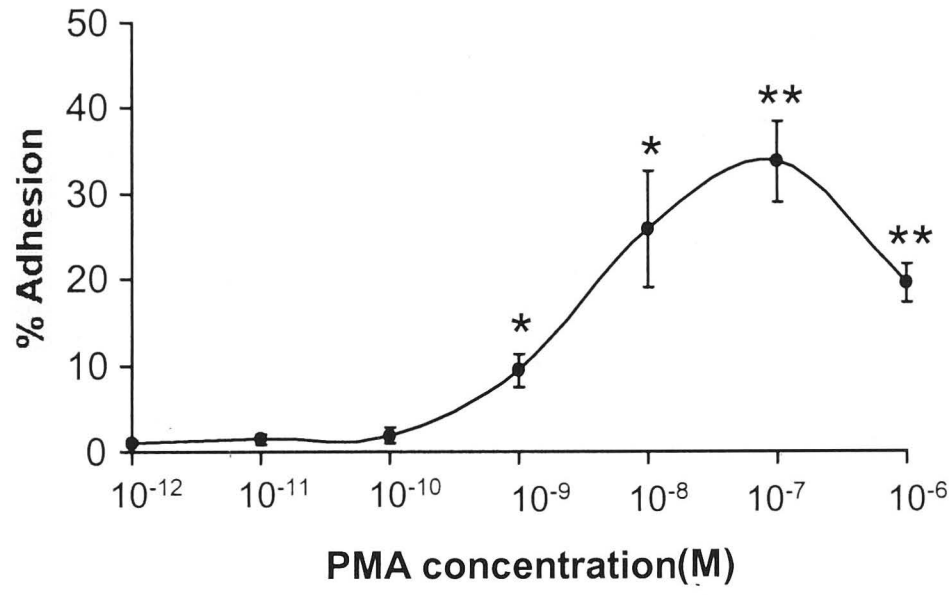
B Degranulation- Release of MBP

Time (min)	Control	PMA
0	-	-
30	-	-
60	-	-
90	-	+
120	-	++
240	-	+++

Data is presented as an index of MBP release.

FIGURE 2.5- Kinetics of mouse eosinophil adhesion (*A*) and degranulation (*B*). Eosinophils were incubated in medium alone or stimulated with 100 nM PMA for the times indicated. *A*, After washing the wells, total EPO in the adherent cells was quantified. Adhesion is represented as the percentage of EPO activity added to each well at t = 0. The results are expressed as the mean ± SEM of 3 separate experiments. Significant differences are $*p < 0.01$ and $**p < 0.001$ from values obtained without stimulus. *B,* Degranulation was monitored by release of MBP into the supernatant and was detected by immunodot blot. The results were scored as a (-) which indicates a background signal and a (+) indicating the detection of MBP with the number of + signs being directly proportional to the color intensity of the dot.

A Adhesion



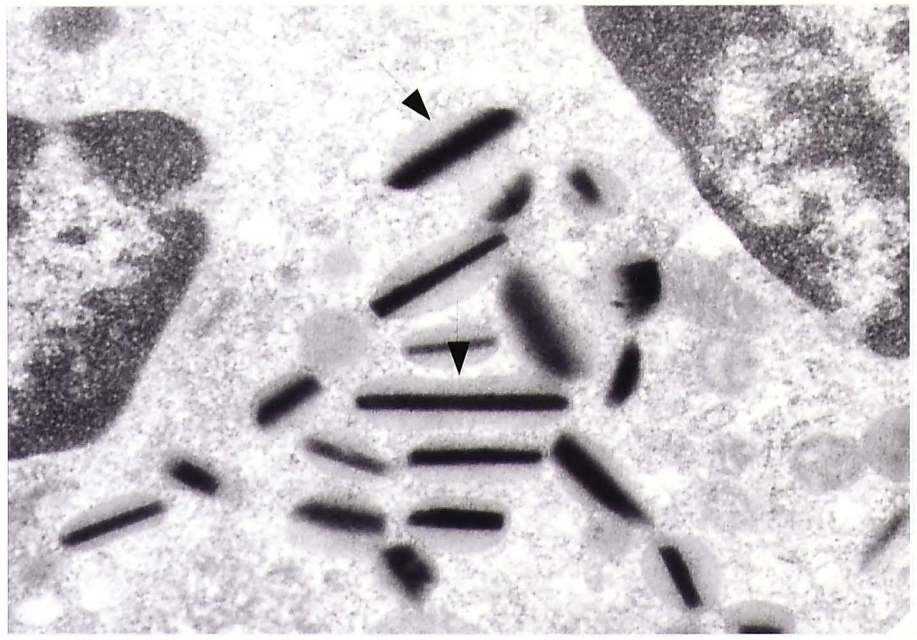
B Degranulation- Release of MBP

[PMA] (M)	MBP release index
0	-
10^{-12}	-
10^{-11}	-
10^{-10}	-
10^{-9}	+
10^{-8}	++
10^{-7}	+++
10^{-6}	++++

FIGURE 2.6- The effect of PMA concentration on eosinophil adhesion (*A*) and degranulation (*B*). Eosinophils were stimulated for 90 min (*A*) or 4 h (*B*) with the indicated concentrations of PMA. *A*, After washing of the wells, total EPO in the adherent cells was determined. Adhesion is expressed as a percentage of EPO added per well at t = 0. The results are expressed as the mean ± SEM of 3 separate experiments. Significant differences are $*p < 0.02$ and $**p < 0.002$ from values obtained without stimulus. *B*, Culture supernatants were recovered and MBP release was determined by immunodot blot. Results were scored as described in fig 2.5.

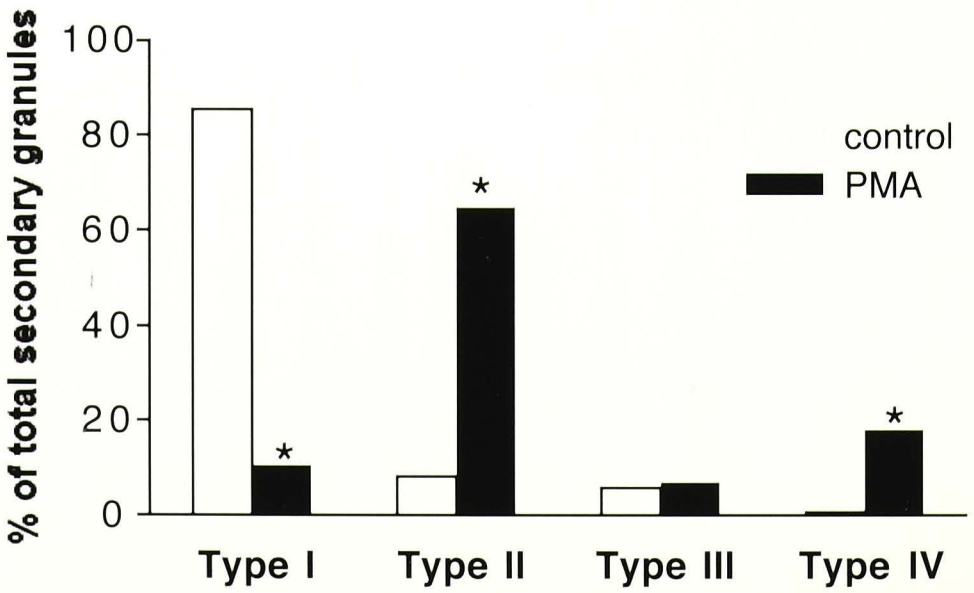
FIGURE 2.7- Electron microscopic analysis of PMA-stimulated eosinophils. *A*, Eosinophils incubated in medium alone show no or few signs of degranulation as the secondary granules display an intact electron dense core surrounded by matrix (type I granule; see arrows). *B*, However, PMA stimulated eosinophils undergo piecemeal degranulation. The secondary granules have an altered phenotype with partial or complete loss of the core but an intact matrix (type II; see black arrow) or partial loss of both the core and matrix (type IV; see white arrow). *C*, Distribution of granule types in eosinophils after PMA stimulation. The secondary granules were counted and categorized in four groups according to granule morphology. The data are presented as the percentage of each granule type from the total secondary granules. Significant differences are *$p < 0.001$ when compared to unstimulated cells. *D*, Effect of PMA on degranulation index. The data is presented as the mean ± SEM.

A

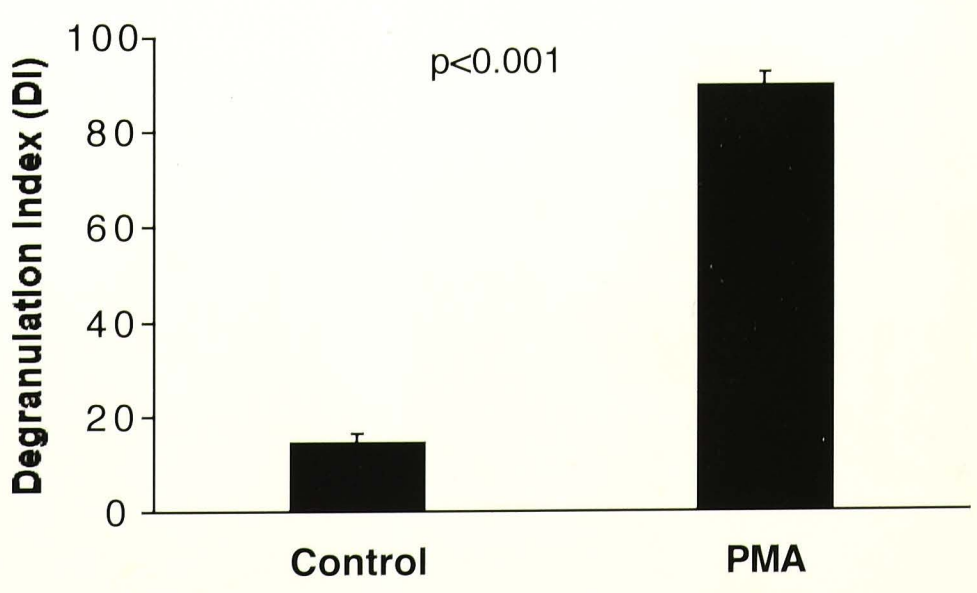


B

C


% of total secondary granules
100
80
60
40
20
0
control
PMA
*
*
*
Type I
Type II
Type III
Type IV

D


Degranulation Index (DI)
100
80
60
40
20
0
p<0.001
Control
PMA

2.3.5 Antigen induces eosinophil degranulation

BALF cells were stimulated with anti-CD3 and OVA to determine if the activation of T cells in respiratory secretions could potentially trigger eosinophil degranulation. Purified eosinophils were also exposed to antigen to determine if antigen uptake or ligation to FcR- Ig complexes could modulate eosinophil activation. PMA was used as a positive control for the induction of degranulation. MBP was released by eosinophils after activation by PMA and exposure to OVA but not by anti-CD3 stimulation. Furthermore, OVA interacted directly with eosinophils inducing degranulation in a highly purified eosinophil population (Table 2.2).

Table 2.2- Eosinophils degranulate in response to antigen.

Cell population	Control	PMA	anti-CD3	OVA
Total BALF cells	-	+	-	+
BALF eosinophils	-	+	N.D.	+

Cellular populations isolated from the airway lumen were cultured in the presence of vehicle control, 100 nM PMA, anti-CD3 or 100 μg of OVA for 4 to 6 h. The culture supernatant was recovered and MBP content was assessed using immunodot blot. The data is represented as a positive signal (+) or a background signal (-). N.D.= not determined.

2.4 DISCUSSION

Mouse models of allergic airways disease have been extensively employed to identify the potential contribution of pulmonary eosinophilia to the pathogenesis of human asthma (Kumar and Foster, 2002). Although many studies have linked eosinophil accumulation in affected tissues with the expression of disease (Foster *et al.*, 1996; Hamelmann *et al.*, 1999a; Tomkinson *et al.*, 2001), interpretation of the significance of these investigations has been limited by the failure to observe extensive activation and degranulation of eosinophils at sites of inflammation (a common feature of human allergic disorders) (Malm-Erjefalt *et al.*, 2001; Stelts *et al.*, 1998). In this investigation we show that eosinophils release granule proteins in the lung via piecemeal degranulation during allergic inflammation of the airways. However, by contrast to asthmatic airways (Erjefalt *et al.*, 1998; Erjefalt *et al.*, 1999), degranulation was primarily compartmentalized to the airway lumen.

Migration of eosinophils into the airway lumen during allergic inflammation resulted in marked activation and the induction of piecemeal degranulation. The ultrastructural features, and in particular, the fraction of granules with a partial loss of both matrix and core (type IV granules) and the degranulation index, strongly indicated a significant loss of granule proteins into the airway lumen in response to antigen inhalation. Indeed, concomitant with ultrastructural changes, significant levels of non-cell-associated EPO were detected in the BALF of allergic mice. Eosinophil recruitment in to the lungs and activation correlate with the induction of AHR supporting earlier studies with rats and primates that directly link eosinophils and their constituents with the induction of disease processes in the lung (Gundel *et al.*, 1991; Uchida *et al.*, 1993). A recent report has also established a temporal-association between the release and persistence of EPO in the airway lumen with the induction and resolution of AHR (Tomkinson *et al.*, 2001). However, it should be noted that AHR is not attenuated in the allergic lung of mice deficient in MBP or EPO, indicating that eosinophils may also regulate airways reactivity independently of these proteins or that other pathways may operate in parallel with granule-induced processes in the allergic lung (Denzler *et al.*, 2001; Denzler *et al.*, 2000).

The release of granule proteins in BALF was not due to sample processing or the result of cellular degenerative processes, since the ultrastructural morphology of BALF eosinophils showed that degranulation occurred via an active process that was reproducibly confined to a distinct percentage of cells. Immunohistochemical studies have also described the deposition of extracellular MBP on the apical surface of

epithelial cells during allergic inflammation of the lungs of mice exposed to *filarial* antigens, indicating that eosinophil degranulation occurs *in vivo* and not *ex-vivo* in the lavage fluid (Mehlotra *et al.*, 1998). Furthermore, recruitment of eosinophils to the airways of naive mice by the overexpression of IL-5 and eotaxin does not subsequently result in eosinophil degranulation in isolated BALF (Mould *et al.*, 2000). We also routinely isolate large numbers of eosinophils from body compartments (peritoneal cavity and blood of IL-5 Tg mice) and have not observed degranulation ex-vivo (Mould *et al.*, 1997).

Although eosinophils were activated in our model, both similarities and differences were observed between mouse and human eosinophils participating in allergic disease. In our mouse model, as in clinical studies, free granule proteins were observed in BALF (Fujimoto *et al.*, 1997; Louis *et al.*, 2000), allergen challenge increased the activation level of circulating eosinophils (Karawajczyk *et al.*, 2000), piecemeal degranulation was the primary mechanism for granule decomposition (Erjefalt *et al.*, 1998; Erjefalt *et al.*, 1999) and apoptotic eosinophils were rare (Erjefalt *et al.*, 1998; Erjefalt *et al.*, 1999). By contrast to human studies (Bozza *et al.*, 1997; Erjefalt *et al.*, 1998; Erjefalt *et al.*, 1999), eosinophils resident in the tissues of the allergic lung of mice displayed only a low level of activation, cytolytic eosinophils were not observed and lipid bodies were rare. These latter observations are in agreement with a previous study (Malm-Erjefalt *et al.*, 2001; Stelts *et al.*, 1998) and suggest that the mechanisms regulating degranulation of mouse and human eosinophils and/or the micro-environment within allergic tissues differs between these species.

Molecules which prime eosinophils can also induce eosinophil degranulation but to a considerably lower degree (Fujisawa *et al.*, 1990; Horie *et al.*, 1996; Koenderman *et al.*, 1996). According to the criteria used in this study many of the eosinophils in the blood and tissues of allergic mice are primed and become further activated to degranulate in the airway lumen. Indeed, eosinophils chronically stimulated with IL-5 in IL-5 Tg mice possessed higher basal levels of activation and attained greater levels of activation after immune stimulation in the airway lumen. The compartmentalization of degranulation primarily to the lumen of the lung may reflect distinct spatial and temporal aspects of acute allergic responses, which are in marked contrast to chronic inflammation , which is observed in human asthma.

In order to characterize the similarities and differences in the regulatory mechanisms of cellular activation between mouse and human eosinophils, we investigated the effect of

PMA on adhesion and degranulation processes. Our data indicate that several features of activation have been conserved between mouse and human eosinophils. Firstly, mouse eosinophils adhered and degranulated in a time- and dose-dependent manner in response to PMA. Although the process of adhesion was slower with mouse eosinophils, like human cells, adhesion preceded degranulation. Thus, adhesion molecules may play a central role in the regulation of mouse eosinophil degranulation as is observed with human cells (Egesten *et al.*, 1993; Horie and Kita, 1994; Kaneko *et al.*, 1995a). Interestingly, human and mouse eosinophils have similar adhesion molecule profiles with both species expressing LFA-1 ($\alpha_L\beta_2$), Mac-1 ($\alpha_M\beta_2$) and VLA-4 ($\alpha_4\beta_1$). These molecules have been implicated in the regulation of various cellular functions including cell-matrix and cell-cell contacts, migration and activation (Horie and Kita, 1994; Kaneko *et al.*, 1995a; Mengelers *et al.*, 1995; Nakajima *et al.*, 1994; Rabb *et al.*, 1994). Secondly, in response to PMA, mouse eosinophils released MBP by piecemeal degranulation, a process widely observed in human eosinophil populations both *in vitro* (in response to various stimuli including PMA) and *in vivo* (Dvorak, 1994; Dvorak *et al.*, 1992; Dvorak *et al.*, 1991; Erjefalt *et al.*, 1998; Erjefalt *et al.*, 1999; Malm-Erjefalt *et al.*, 2001). Piecemeal degranulation provides a mechanism for the preferential release of granule proteins (Tomassini *et al.*, 1991). In response to PMA, mouse eosinophils appear to release, preferentially, proteins associated with the core, notably MBP. After activation, the largest proportion of secondary granules progressed from type I to type II granules (fig. 2.7).

In addition to piecemeal degranulation, cell death contributes to the release of MBP. Prolonged exposure of eosinophils to PMA resulted in cellular death as depicted by the loss of plasma membrane integrity and clear spaces within the cells. Notably, cellular death was confined to the adherent population as the non-adherent eosinophils remained viable throughout the experiment according to the trypan blue exclusion assay. This result is in agreement with a previous report which examined the viability of human eosinophils in response to sIgA- and IgG-coated beads (Weiler *et al.*, 1996). Since cell adhesion is instrumental in the process of degranulation, only adherent cells secrete cytotoxic cationic proteins which once bound to the cell surface induce cell death. Another potential explanation is that cell adhesion serves as a signal to induce cytolysis and permit the cell to secrete large quantities of mediators rapidly. Finally, the high concentration of PMA used in these experiments induced the adhesion of mouse eosinophils. Thus, it is unlikely that the release of MBP by mouse eosinophils is a passive process due to cellular death but more likely to be the result of a highly

regulated process involving cell adhesion, piecemeal degranulation and culminating in cytolysis.

Although, the kinetics of adhesion and degranulation and the mechanism for the release of granule contents (piecemeal degranulation) were similar to human cells, higher concentrations of PMA were required to induce these responses (Horie and Kita, 1994; Malm-Erjefalt *et al.*, 2001; Reimert *et al.*, 1998). The optimal concentration of PMA required to stimulate both adhesion and degranulation would appear to be significantly different between species. Mouse eosinophil activation occurred at concentrations as low as 1 nM but only reached a maximum at 100 nM. In contrast, human eosinophils only require 1.5 nM of PMA to secrete maximal amounts of granule proteins (Horie and Kita, 1994; Reimert *et al.*, 1998). The threshold of responsiveness highlights an important difference between human and mouse eosinophils (Malm-Erjefalt *et al.*, 2001).

To determine a potential mechanism regulating mouse eosinophil activation *in vivo*, eosinophils were cultured in the presence of various stimuli. Eosinophils can sequester antigen in the allergic lung and activate Th2 cells. We therefore investigated the possibility that factors associated with these processes promote degranulation (MacKenzie *et al.*, 2001; Mattes *et al.*, 2002; Shi *et al.*, 2000). Th2 cell factors in association with cognate antigen have also implicated in regulating eosinophil degranulation in response to *Nippostrongylus brasiliensis* infection in mice (Shinkai *et al.*, 2002). We were particularly interested in the effect of OVA as a potential secretagogue of airway eosinophils because it is not only a prominent molecule in the airway lumen in this experimental model but also because antigens activate eosinophils recovered from atopic patients (Kaneko *et al.*, 1995b; MacKenzie *et al.*, 2001; Tomassini *et al.*, 1991). The activation of T cells by anti-CD3 in cultured unfractionated BALF cells from allergic mice did not trigger eosinophil degranulation. However, incubation of both unfractionated BALF cells or purified eosinophils with OVA led to the release of MBP. The induction of degranulation by antigen is thought to occur by the binding to cytophilic immunoglobulins at the surface of the cell (Kaneko *et al.*, 1995b; Tomassini *et al.*, 1991). Thus, the release of granule proteins in the airway lumen of allergic mice may be achieved through the interaction between Fc receptors, immunoglobulins and OVA at the surface of eosinophils. Interestingly, eosinophils recruited to the lung by the local expression of exogenous IL-5 and eotaxin in naive mice could only induce AHR after the concomitant delivery of OVA to the airways and this was coincident with the induction of MBP release from eosinophils (Mould *et al.*,

2000). Antigen is primarily localized to the airway lumen during acute inflammatory reactions in mouse models (MacKenzie *et al.*, 2001) and it is tempting to speculate that this phenomena results in eosinophil degranulation occurring primarily in this compartment. The dose and dispersal of antigen within the lung may be an important determinants of where and if further eosinophil degranulation occurs in mouse models of allergic lung disease. Indeed, in studies where eosinophil degranulation was not observed in the allergic lung of mice only modest levels of antigen were delivered to the lung, with at markedly reduced exposure times by comparison to our investigation (Malm-Erjefalt *et al.*, 2001; Stelts *et al.*, 1998).

In summary, we have provided evidence for eosinophil degranulation in our model of allergic airways disease and linked eosinophil activation in the airway lumen to the induction of AHR. EPO was detected in the BALF of sensitized mice and electron microscopy of eosinophils depicted morphological changes characteristic of piecemeal degranulation. Although the eosinophils in the airway submucosa were not releasing large amounts of granule contents, these cells were activated by comparison to control and bone marrow-derived eosinophils. However, eosinophils were highly activated in the airway lumen. Our *in vitro* studies indicate that like human cells, mouse eosinophil activation follows adhesion, but these cells are more resistant to activation by PMA. The differences (degree and site of activation) observed *in vivo* may be due, in part, to the higher thresholds for activation of mouse eosinophils and the acute nature of the disease process in mice compared to humans. Understanding the mechanisms regulating mouse eosinophil degranulation will help improve our interpretation of mouse models of allergic airways disease and enhance their value as correlates of human disease.

3

Purification of eosinophils from bone marrow, blood and BALF of allergic mice using light polarization properties of cells

3.1 INTRODUCTION

Several signals are required to cooperate in order for eosinophils to influence the pathogenesis of allergic diseases. In particular, IL-3, IL-5 and GM-CSF direct the proliferation and differentiation of eosinophils in the bone marrow (Lopez *et al.*, 1987; Mayer *et al.*, 1989; Metcalf *et al.*, 1986; Sanderson *et al.*, 1985), the eotaxin family serves as the primary chemotactic signals within tissues (Forssmann *et al.*, 1997; Jose *et al.*, 1994; Kitaura *et al.*, 1999; Shinkai *et al.*, 1999) and immunoglobulins, lipid mediators and complement proteins activate eosinophils (Abu-Ghazaleh *et al.*, 1989; Horie *et al.*, 1996; Kaneko *et al.*, 1995b; Khalife *et al.*, 1986; Khalife *et al.*, 1985; Kroegel *et al.*, 1989; Takafuji *et al.*, 1994). These signaling networks lead to the release of pro-inflammatory mediators by eosinophils which modulate the inflammatory response and tissue function (Gundel *et al.*, 1991; Hisamatsu *et al.*, 1990). The temporal and spatial regulation of these signaling molecules produces eosinophil populations with different phenotypes. Indeed, in the previous Chapter, it was demonstrated that the activation status of eosinophils increases as the cells migrate to sites of inflammation. The ability to isolate eosinophils from the bone marrow, blood and BALF of allergic mice would permit the complete characterization of each eosinophil population and provide a platform to identify molecules which regulate the differentiation, migration and activation of eosinophils. The information may enable the discovery of new therapies to prevent eosinophil recruitment and activation within allergic diseases.

Several methods currently exist to isolate specific eosinophil populations from humans and other species. These protocols were analyzed to determine whether they could be adapted to the purification of eosinophils from the bone marrow, blood and BALF of allergic mice. Firstly, eosinophils from human blood may be purified by either FACS or using immunomagnetic beads on the basis of CD16 negativity (Gopinath and Nutman, 1997; Hansel *et al.*, 1991b; Thurau *et al.*, 1996). In the first step, lymphocytes and monocytes are separated from the granulocytes by means of a density gradient. Subsequently, neutrophils can be distinguished from eosinophils due to the differential surface-expression of CD16 in myeloid cells. These methods are rapid and use a negative selection procedure minimizing effects on eosinophils. Unfortunately, a similar method could not be adapted for the isolation of mouse eosinophils because CD16 is constitutively expressed on the surface of mouse eosinophils (de Andres *et al.*, 1994) and the current antibodies commercially available against mouse FcγR recognize both CD16 and CD32 (Serrander *et al.*, 2000). The latter molecule is widely expressed in eosinophils (de Andres *et al.*, 1994; Kim *et al.*, 1999).

Recently, methods have been developed to purify eosinophils from mice. Shinawaga and Anderson (Shinagawa and Anderson, 2000) have described a purification method of mouse eosinophils using immunomagnetic beads coated with antibodies directed against CD4 and CD8 present on lymphocytes, and lectins which preferentially bind to macrophages. However, this method is restricted to eosinophils isolated from the airway lumen of allergic mice. It might be difficult to convert this method for the isolation of eosinophils from the bone marrow and blood because, in contrast to BALF, these samples possess high levels of neutrophils requiring a method to distinguish and separate the two cell populations. Other researchers have described an alternative immunomagnetic method for the isolation of eosinophils from the spleen and blood of IL-5 Tg mice (Rothenberg *et al.*, 1995; Teixeira *et al.*, 1997). However, these methods do not separate the two granulocyte populations and rely primarily on the altered neutrophil to eosinophil ratio in the IL-5 Tg mice to overcome the problem. Hence, the primary hurdle to adapt any purification method to eosinophils present in different compartments of allergic mice is the ability to differentiate the granulocyte populations.

While CD16 cannot be used as a marker to differentiate murine granulocytes as in the case of human cells, other surface markers could be used to distinguish mouse eosinophils from neutrophils. Thurau and colleagues (Thurau *et al.*, 1996) successfully adapted CD49d expression for this purpose. Additional markers include C-C chemokine receptor (CCR)-3 and IL-5Rα. However, methods based on these molecules would involve a positive selection of eosinophils and ligation of these receptors with an antibody may activate intracellular signaling pathways. In order to recover eosinophils which represent as closely as possible the *in vivo* status of the cells, a negative selection process is required.

Mouse eosinophils can also be purified from a complex mixture of cells by FACS using FSC vs. SSC and light polarization properties of cells as parameters (Mould *et al.*, 1997). Light polarization reflects the complexity of a cell including the granule content. Since the composition of granules is different between granulocyte populations, the cells might polarize light differentially permitting the distinction of each leukocyte.

In this chapter, we determined whether light polarization was an adequate parameter to distinguish each leukocyte population including granulocytes. Indeed, eosinophils could be isolated from the bone marrow, blood and BALF of allergic mice using the FACS-based method. Furthermore, preliminary investigations demonstrated the potential of

DNA micro-arrays and subtractive cloning in the identification of differentially expressed genes from eosinophils isolated from difference compartments. Cytokines and their receptors, chemokine receptors and cytotoxic cationic proteins were differentially expressed in bone marrow and BALF eosinophils.

3.2 MATERIALS AND METHODS

3.2.1 Animals

See section 2.2.1

3.2.2 Induction of allergic airways disease

See section 2.2.2

3.2.3 Collection of bone marrow, blood and BALF cells

Eosinophils were purified from the cellular populations present in the bone marrow, peripheral blood and BALF. The blood was collected from the femur artery of an anesthetized mouse into a tube containing heparin in PBS. The bone marrow cells were obtained from the femur by cutting each extremity of the bone and rinsing the cavity twice with 1 ml of PBS. The eosinophils present in the lumen of the airways were recovered by instilling in the lungs, via a catheter, 750 µl of PBS thrice. The samples from several animals were pooled to ensure a sufficient number of cells was available for purification.

3.2.4 Sample preparation for FACS analysis

The red blood cells from all fractions were lysed using an ammonium chloride solution (0.16 M NH_4Cl, 0.17 M Tris pH 7.2). The cells were resuspended in PBS containing 1% fetal calf serum (CSL Ltd., Parkville, Australia) and debris was removed by passing the suspension through FACS gauze.

3.2.5 Purification of eosinophils by FACS

Eosinophil purification was performed using a FACStar Plus instrument equipped with a 488 nm Coherent Innova 90 laser (200 mW). Forward angle, 90° light scattering and light polarization were measured. For the latter, a filter set was installed into the FL1 (Polarizer-P-horizontal) and FL2 (Polarizer-S-vertical) channels. Eosinophils were selected using specific electronic gates and collected in PBS containing 5% fetal calf serum. The purity of the population was assessed by light microscopy after staining cytospins with either May-Grünwald-Giemsa dyes.

3.2.6 May-Grünwald-Giemsa staining of cells

Purified cells were cytospun onto glass slides, air dried and fixed in ethanol for 15 min prior to staining. Subsequently, the samples were successively stained for 5 min in

May-Grünwald solution (0.15 % May-Grünwald, 50% methanol dissolved in a commercial buffer (buffer tablets pH 6.4 for staining of blood smears according to the Weise protocol, BDH Lab Supplies, Poole, England)) and 10 min in Giemsa stain (0.125% Giemsa, 7.25% glycerol, 8.3% methanol dissolved in the Weise buffer). To wash away the excess stain, cells were destained in Weise buffer for 10 min. The specimens were air dried and mounted for visualization by light microscopy.

3.2.7 Adhesion assay

See section 2.2.9

3.2.8 Degranulation assay

See section 2.2.10

3.2.9 EPO assay

See section 2.2.11

3.2.10 MBP dot blot

See section 2.2.11

3.2.11 Purification of RNA

Total RNA was extracted from the purified eosinophils using a one-step solution based on a method devised by Chomczynski *et al.* (Chomczynski and Sacchi, 1987). The cells were lysed in RNAzol^{TM} B (Geneworks Pty. Ltd., Adelaide, SA, Australia) at $2.5x10^6$/ml. After the first phenol-chloroform extraction, the aqueous phase was mixed with an equal volume of RNAzol^{TM} B and 1/5th volume of chloroform. The RNA was finally recovered by precipitation using GlycoBlue^{TM} (Ambion Inc., Texas, USA) as carrier at a concentration of 30-50 μg/ml and an equal volume of isopropanol. The RNA pellet was washed once with 75% ethanol before being dissolved in diethyl pyrocarbonate (DEPC)-water. The RNA was quantitated by spectrophotometric analysis whereby the absorbance at 260 nm was measured.

3.2.12 RNA gel electrophoresis

RNA samples were separated by gel electrophoresis using a 1.2% agarose gel prepared in 1X FA buffer (20 mM 3-[N-morpholino]propanesulfonic acid, 5 mM sodium acetate, 1 mM EDTA pH 7.0) supplemented with 0.7% formaldehyde and 0.1 μg/ml ethidium bromide. The gel was equilibrated in running buffer (1X FA buffer, 0.7%

formaldehyde) for 30 min. RNA samples (1-2 μg) were diluted with 0.2 volumes of loading buffer (4X FA buffer, 2.7% formaldehyde, 20% glycerol, 31% formamide, 0.25% bromphenol blue), heated to 65°C for 3-5 min and subsequently chilled on ice. The entire sample was loaded onto the gel. A constant voltage of 100 V was applied during the electrophoresis procedure.

3.2.13 RT-PCR

The first-strand complementary (c)DNA synthesis was performed using the SMART technology developed by ClonTech Laboratories (Palo Alto, CA, USA). Total RNA (1 μg) was reverse transcribed according to the manufacturer's protocols. The RNase H$^-$ reverse transcriptase Superscript^{TM} II (Gibco BRL, Mulgrave, Vic, Australia) was employed in all reactions.

Polymerase chain reaction (PCR) amplification of specific genes was performed according to standard protocols (Ausubel *et al.*, 1998). The sequence of all gene-specific primers are defined in Table 3.1. The cDNA was amplified using an initial denaturation step of 2 minutes at 95°C followed by 20 to 35 cycles of 15 seconds at 95°C, 15 seconds at 50°C and 90 seconds at 72°C. A final extension at 72°C was performed for 7 minutes. The PCR products were separated by electrophoresis in a 2% agarose gel and visualized using either ethidium bromide or Vistra^{TM} Green (1:10000; Amersham Pharmacia Biotech Pty. Ltd., Sydney, NSW, Australia) as fluorescent marker. The image of the gel was recorded using a Fluorescent Image Analyser FLA-3000 (Fuji Film, Japan) and analyzed with Imagegauge 3.11 software. The intensity of the cDNA bands were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Table 3.1- Sequence of gene-specific primers utilized for expression profiling of eosinophils.

Primer	Sequence
GAPDH, sence	5′ ACC ACA GTC CAT GCC ATC AC 3′
GAPDH, anti-sense	5′ TCC ACC ACC CTG TTG CTG TA 3′
IL-4, sense	5′ GAA TGT ACC AGG AGC CAT ATC 3′
IL-4, anti-sense	5′ CTC AGT ACT ACG AGT AAT CCA 3′
IL-5, sense	5′ GAC AAG CAA TGA GAC ACG ATG AGG 3′
IL-5 anti-sense	5′ GAA CTC TGC AGG TAA TCC AGG 3′
IL-12, sense	5′ CGT GCT CAT GGC TGG TGC AAA G 3′
IL-12, anti-sense	5′ CTT CAT CTG CAA GTT CTT GGG C 3′
IL-13, sense	5′ CTC CCT CTG ACC CTT AAG GAG 3′
IL-13,anti-sense	5′ GAA GGG GCC GTG GCG AAA CAG 3′
IFN-γ, sense	5′ AAC GCT ACA CAC TGC ATC TTG G 3′
IFN-γ, anti-sense	5′ GAC TTC AAA GAG TCT GAG G 3′
CCR-3, sense	5′ AAG TAC AGG AAG CTA CAA ATT ATG 3′
CCR-3, anti-sense	5′ AGC AGA GTT TTA ATG ATT CCT GAG 3′
CCR-4, sense	5′ ATC GTG CAC GCG GTA TTC TCC 3′
CCR-4, anti-sense	5′ GAC GGG GTT AAG GCA GCA GTG A 3′
CxCR-3, sense	5′ TCA TCT TCC TGT CAG CCA GC 3′
CxCR-3, anti-sense	5′ CAC CAC CAC CAC CAC CAC TA 3′
MBP, sense	5′ AAG GAA GAA GGT TCT GGA AGT GA 3′
MBP, anti-sense	5′ CCA GTA TGC AAA ATT CCA AGA ACT 3′
IL-5Rα, sense	5′ ATT AAA GCC ACT GGA TTA GCT CAA 3′
IL-5Rα, anti-sense	5′ ATT GCT TTC AAT TTC CAC TGT GAC 3′
IL-4Rα, sense	5′ GCC CCA GTG GTA ATG TGA AGC CCC 3′
IL-4Rα, anti-sense	5′ CCC AGA GGG GCA CCT GTG CAT CC 3′
IL-13Rα1, sense	5′ ACA GAA GTT CAG CCA CCT GTG ACG 3′
IL-13Rα1, anti-sense	5′ GGT GGA GTT TTG CTC CTT ACC TAT AC 3′
IL-13Rα2, sense	5′ ATG GCT TTT GTG CAT ATC AGA TGC 3′
IL-13Rα2, anti-sense	5′ GGT GTG CTC CAT TTC ATT CTA ATA TC 3′

3.3 RESULTS

3.3.1 Eosinophilia reached maximal levels on day 31

The kinetics for the development of an eosinophilia in the bone marrow, peripheral blood and BALF within the mouse model of allergic airways disease were measured in order to possess optimal cell numbers for purification purposes. Eosinophil numbers in all compartments increased slightly after each of the first 3 challenges. However, the exposure of mice to antigen on day 30 led to a sharp increase in eosinophilia in all compartments (Fig. 3.1). Additional exposures of the mice to OVA either led to a decrease in eosinophil levels or only minor increases. Hence, cellular populations isolated from the bone marrow, blood and BALF of allergic mice on day 31 were utilized for the isolation of eosinophils.

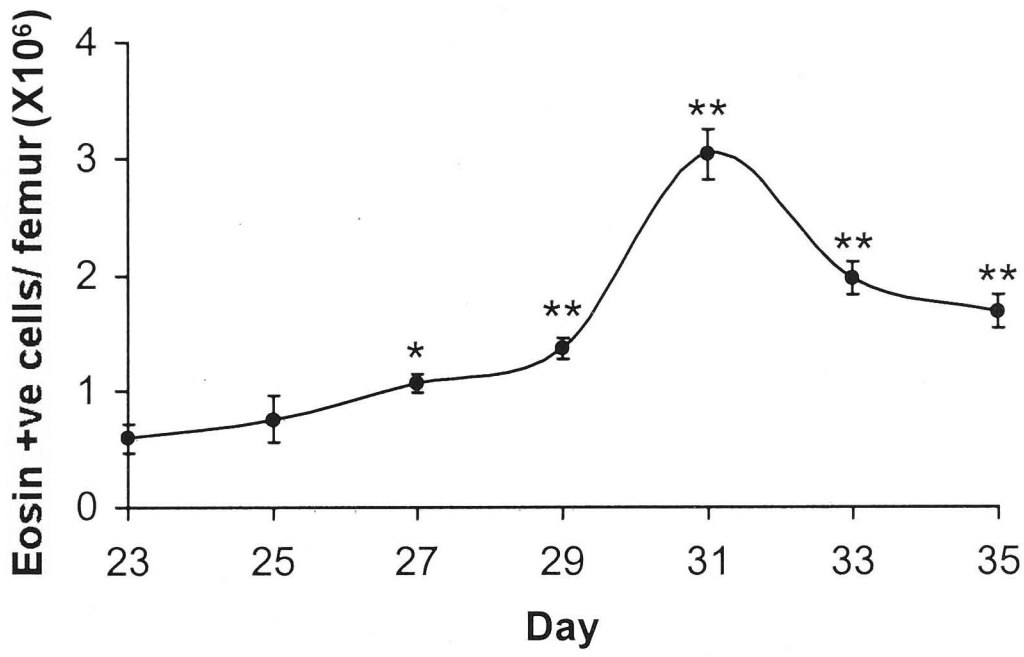
3.3.2 Amplification of a cell population with high side scatter and light polarization properties during allergy

During allergic airways disease, the presence of lymphocytes, neutrophils and monocytes/macrophages also increased in the different compartments (data not shown). However, only the eosinophil population displayed a marked increase as a fraction of the entire leukocyte population (Fig. 3.2). This effect was also very pronounced. In particular, while eosinophils only consisted of less than 1% of all leukocytes in the BALF under normal conditions, the fraction of eosinophils increased to approximately 70% after antigen challenge. Hence, by comparing the profile of leukocyte populations on FACS under normal and allergic conditions, it may be possible to identify the eosinophils.

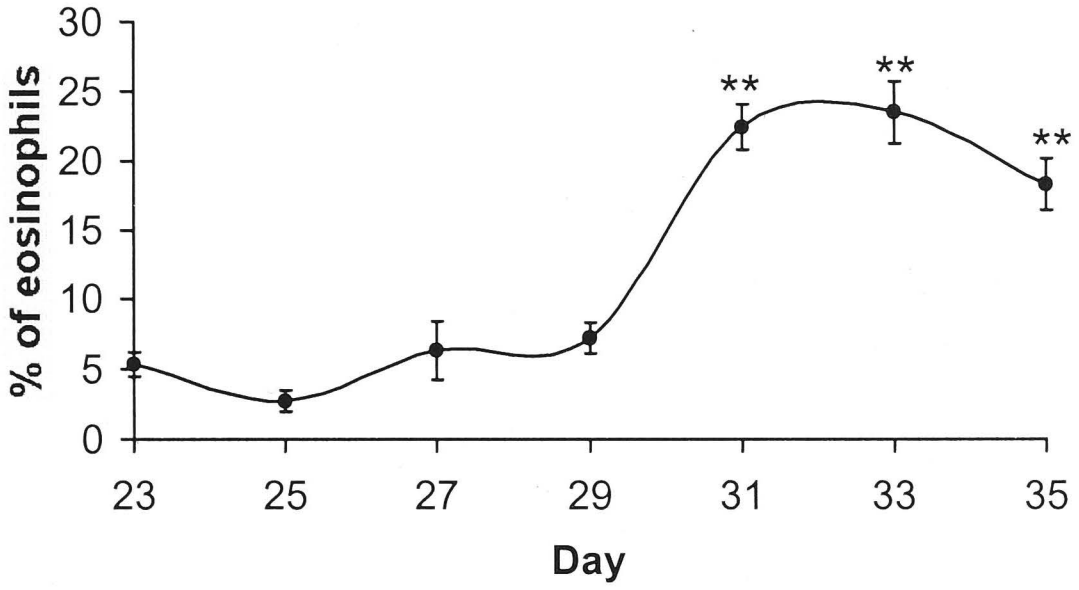
Purification of eosinophil populations on the basis of the FSC vs. SSC plot and light polarization properties had previously been described (Mould *et al.*, 1997). Gates for the purification of eosinophils by FACS using FSC vs. SSC and light polarization were set for the BALF population since eosinophils are the predominant leukocyte (70%) and granulocyte (>90%). In both the FSC vs. SSC and light polarization plots, a distinct population corresponding to the eosinophil was identified (Fig. 3.3-3.5). Unfortunately, these gates could not be directly used for the isolation of eosinophils from the bone marrow and blood because neutrophils have a similar profile to eosinophils. To distinguish the two populations, plots obtained from normal mice were compared to allergic mice. In both the bone marrow and blood samples, an increase was observed in a population displaying high SSC and light polarization properties (Fig. 3.3- 3.4) and

FIGURE 3.1- Kinetics for the generation of an eosinophilia in the bone marrow, blood and BALF during a murine model of allergic airways disease. BALB/c mice were sensitized to OVA and subsequently, challenged with antigen. The eosinophilia in each compartment was determined 24 hours after antigen exposure. A, Bone marrow eosinophilia. Since both eosinophil progenitors and mature cells are present in the bone marrow, all eosin positive cells were included in the sample. The data is presented as the average eosin positive cells/ femur ± SEM (n = 4-8 mice per group). B, Eosinophils in the peripheral blood. The data is expressed as the percentage of eosinophils in the blood (mean ± SEM, n = 4-8 mice per group). C, Pulmonary eosinophilia. The data is presented as the total number of eosinophils in BALF (mean ± SEM, n = 4-8 mice per group). Significant differences are $*p < 0.05$ and $**p < 0.001$.

A Bone Marrow



B Blood



C BALF

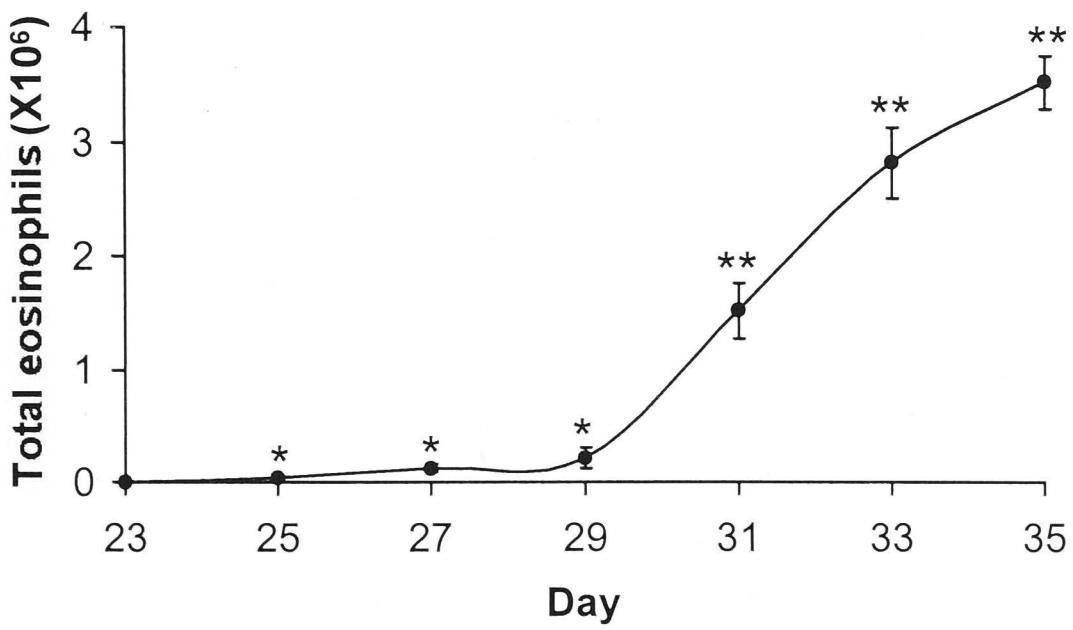
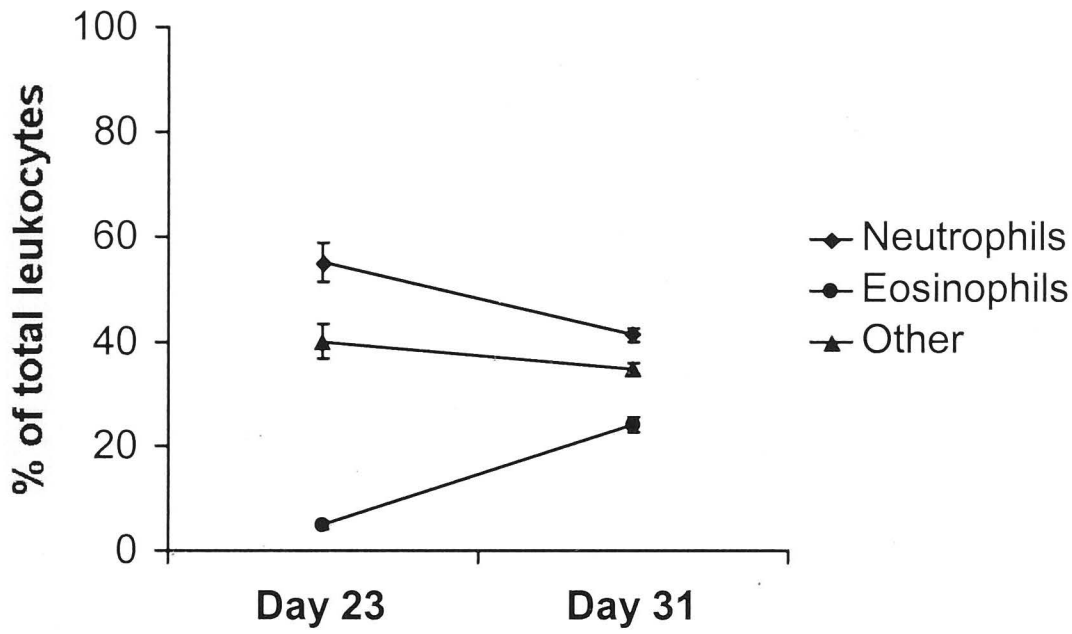
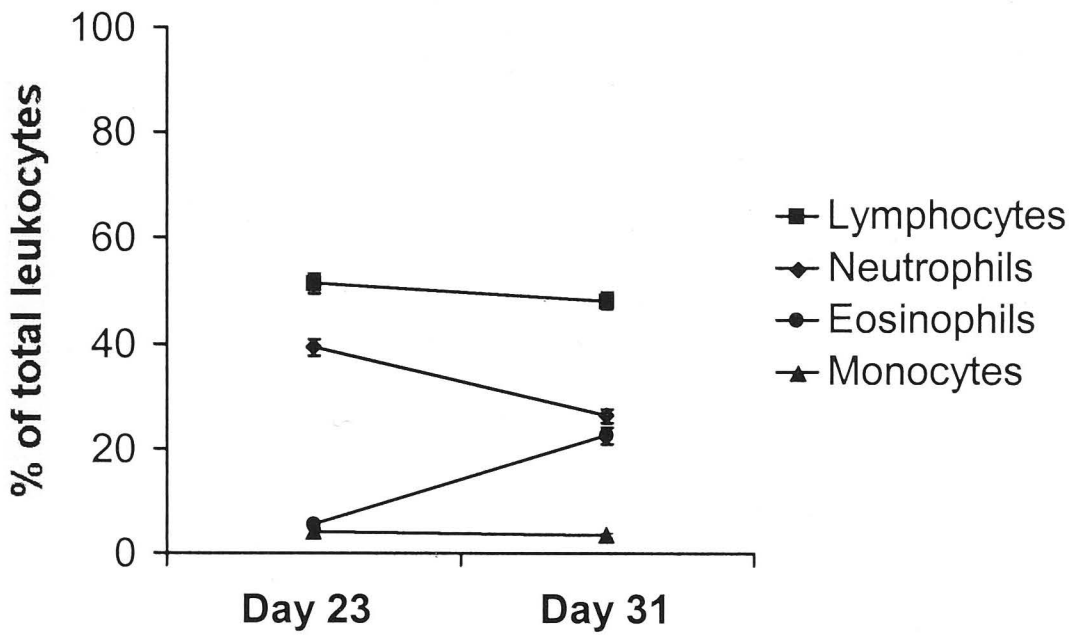


FIGURE 3.2- The eosinophil is the only leukocyte population depicting an increase as a proportion of all leukocytes. The data is presented as the percentage of each leukocyte among the total cellular populations in bone marrow (A), peripheral blood (B) and BALF (C) (mean ± SEM, n = 4-8 mice per group). For clarity, only the data collected before antigen challenge (day 23) and after the development of a strong inflammatory response (day 31) is presented.

A Bone Marrow



B Blood



C BALF

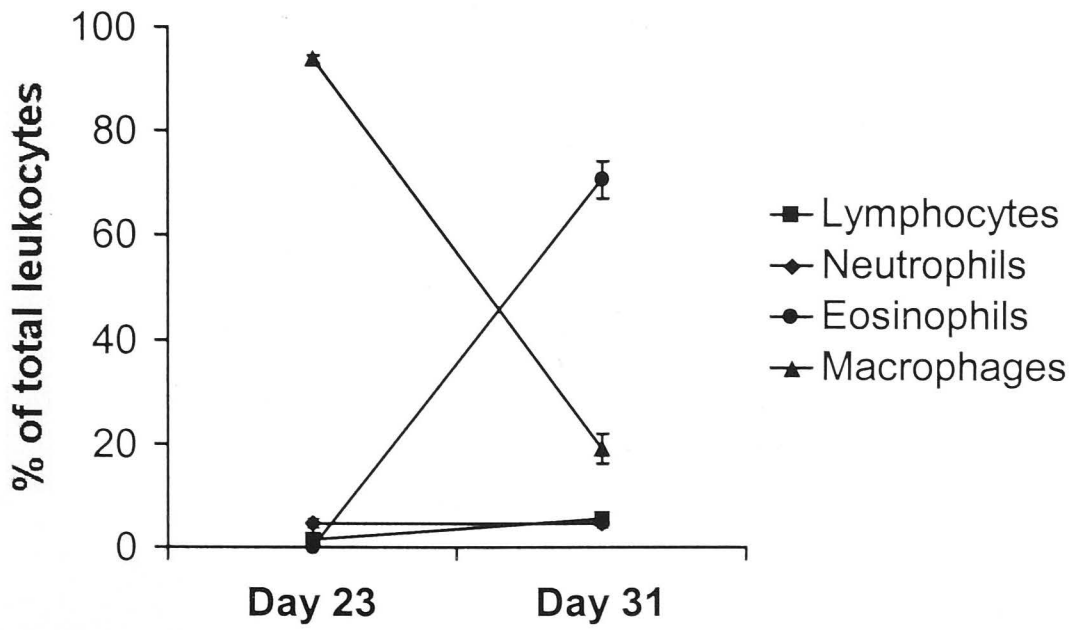
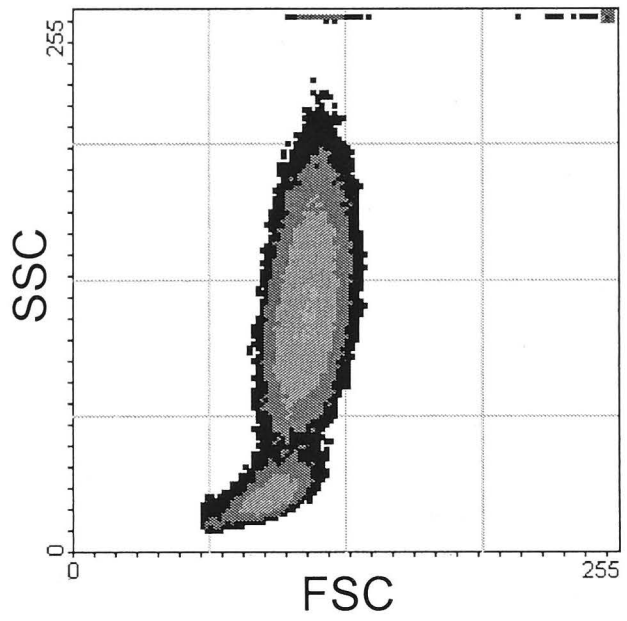
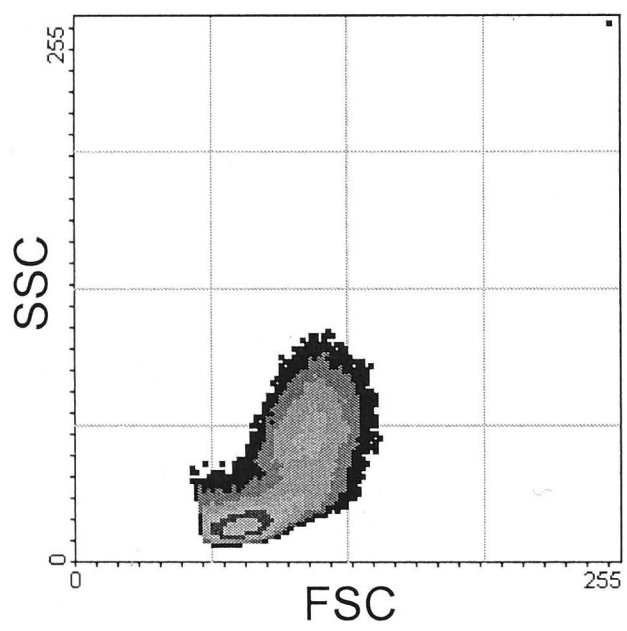


FIGURE 3.3- A leukocyte population with a high side scatter is amplified during allergy. Cells were isolated from bone marrow and blood of control mice and bone marrow, blood and BALF of allergic mice. The FSC vs. SSC profile of each population was analyzed by FACS. Analysis of BALF (A) was used as an initial indicator since BALF consists of 70% eosinophils. In the bone marrow (B, control; C, allergic) and blood (D, control; E, allergic), a population with a higher SSC, indicated by the arrow, is amplified during allergy.

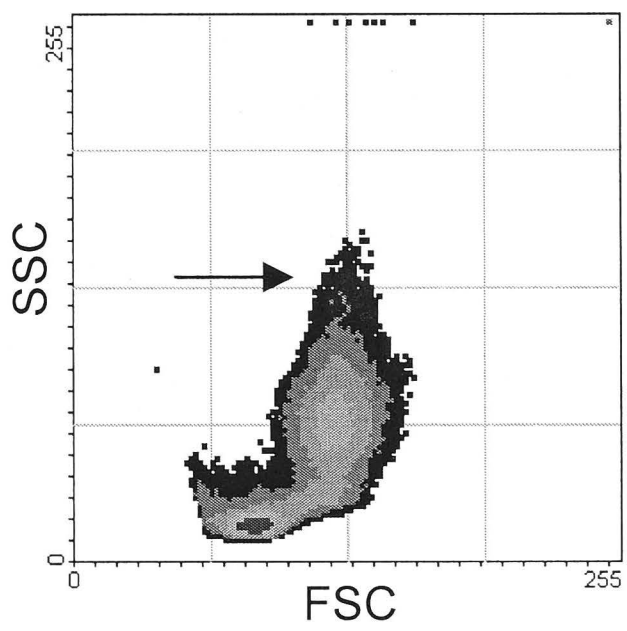
A BALF



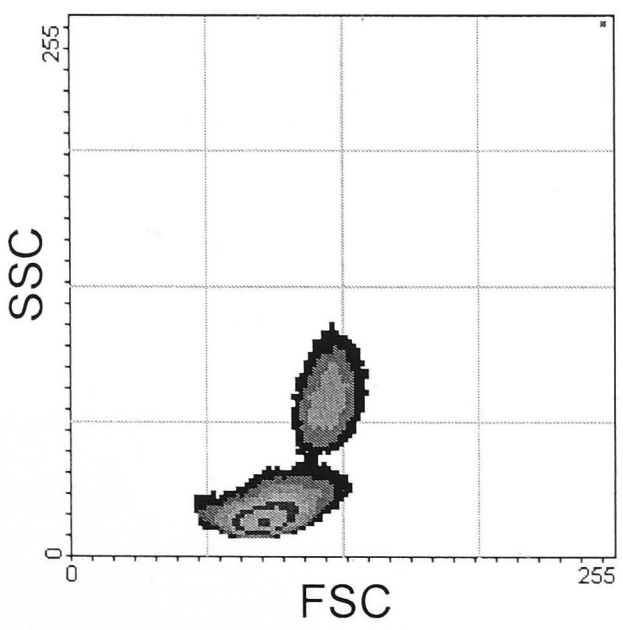
B Naive- Bone Marrow



C Allergic- Bone Marrow



D Naive- Blood



E Allergic- Blood

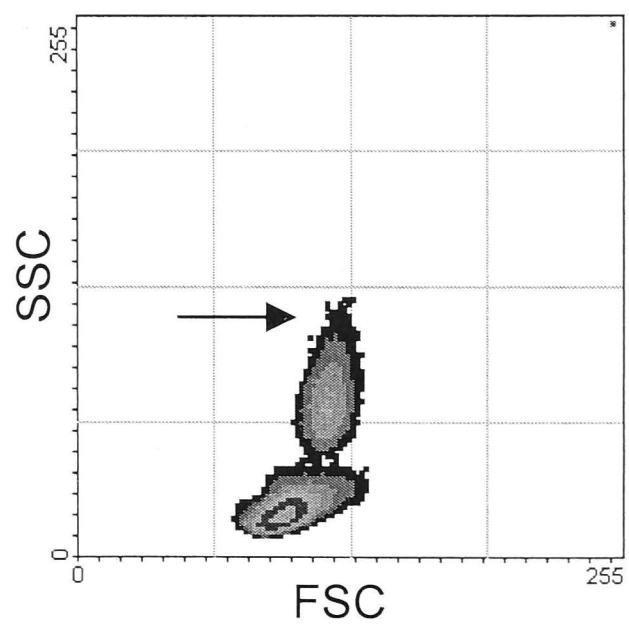
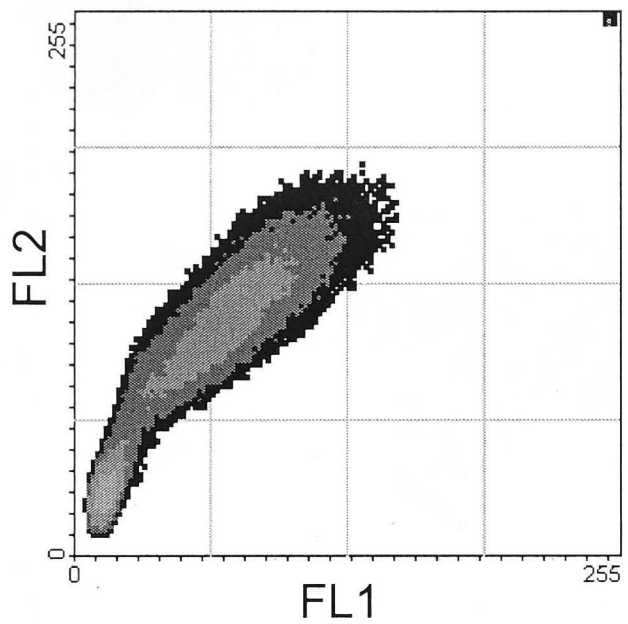
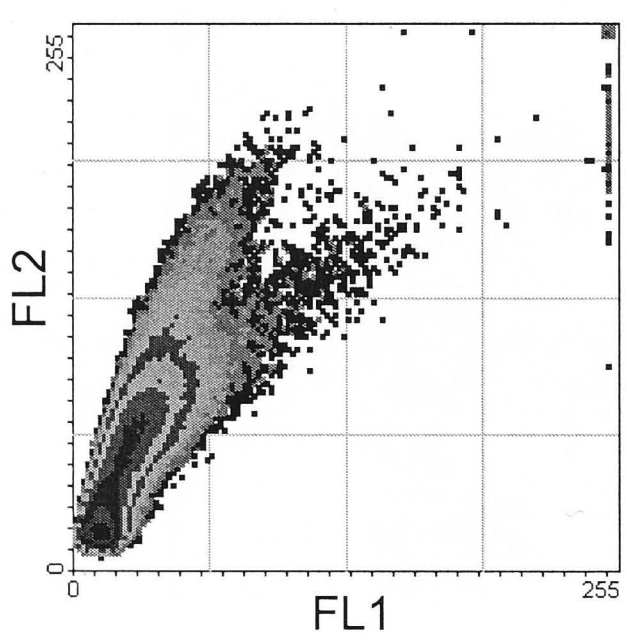


FIGURE 3.4- A leukocyte population capable of polarizing light is amplified during allergy. Cells were isolated from bone marrow and blood of control mice and bone marrow, blood and BALF of allergic mice. A filter set which permits the measurement of light polarization was introduced in the channels FL-1 and FL-2 of the FACS instrument. Light polarization by each population was recorded. Analysis of BALF (A) was used as an initial indicator since BALF consists of 70% eosinophils. In the bone marrow (B, control; C, allergic) and blood (D, control; E, allergic), a population which polarizes light, indicated by the arrow, is amplified during allergy.

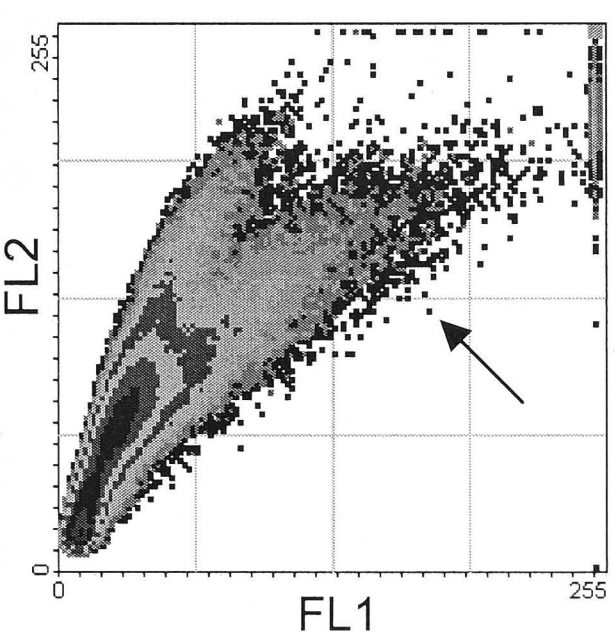
A BALF



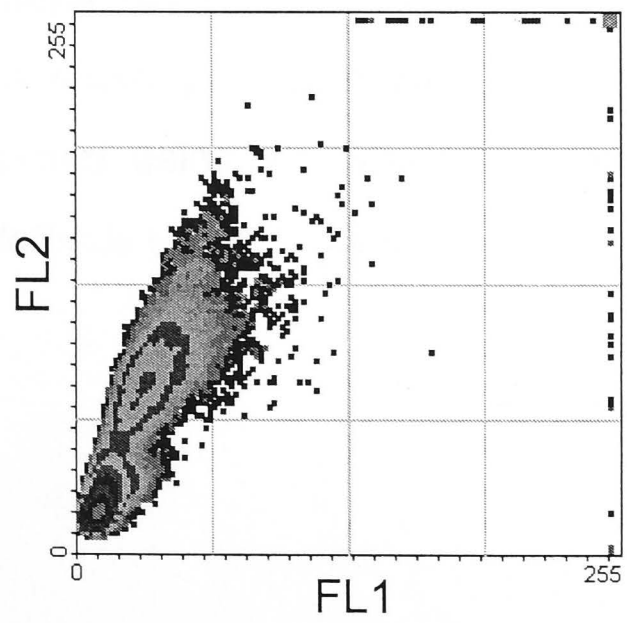
B Naive- Bone Marrow



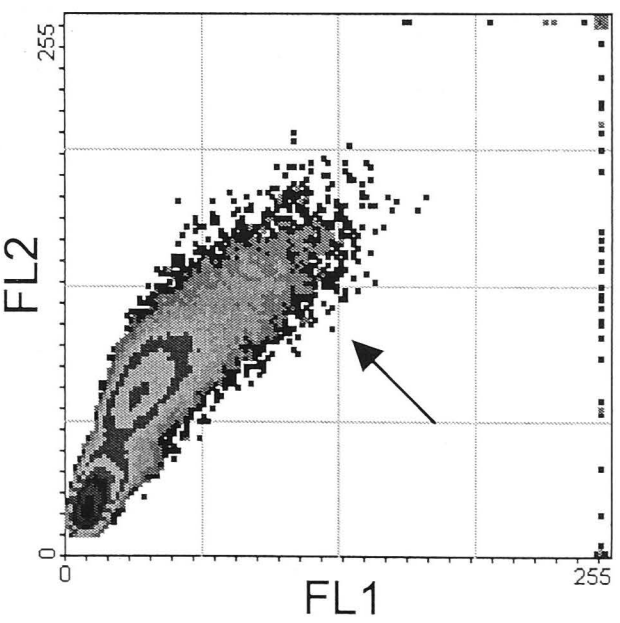
C Allergic- Bone Marrow



D Naive- Blood



E Allergic- Blood



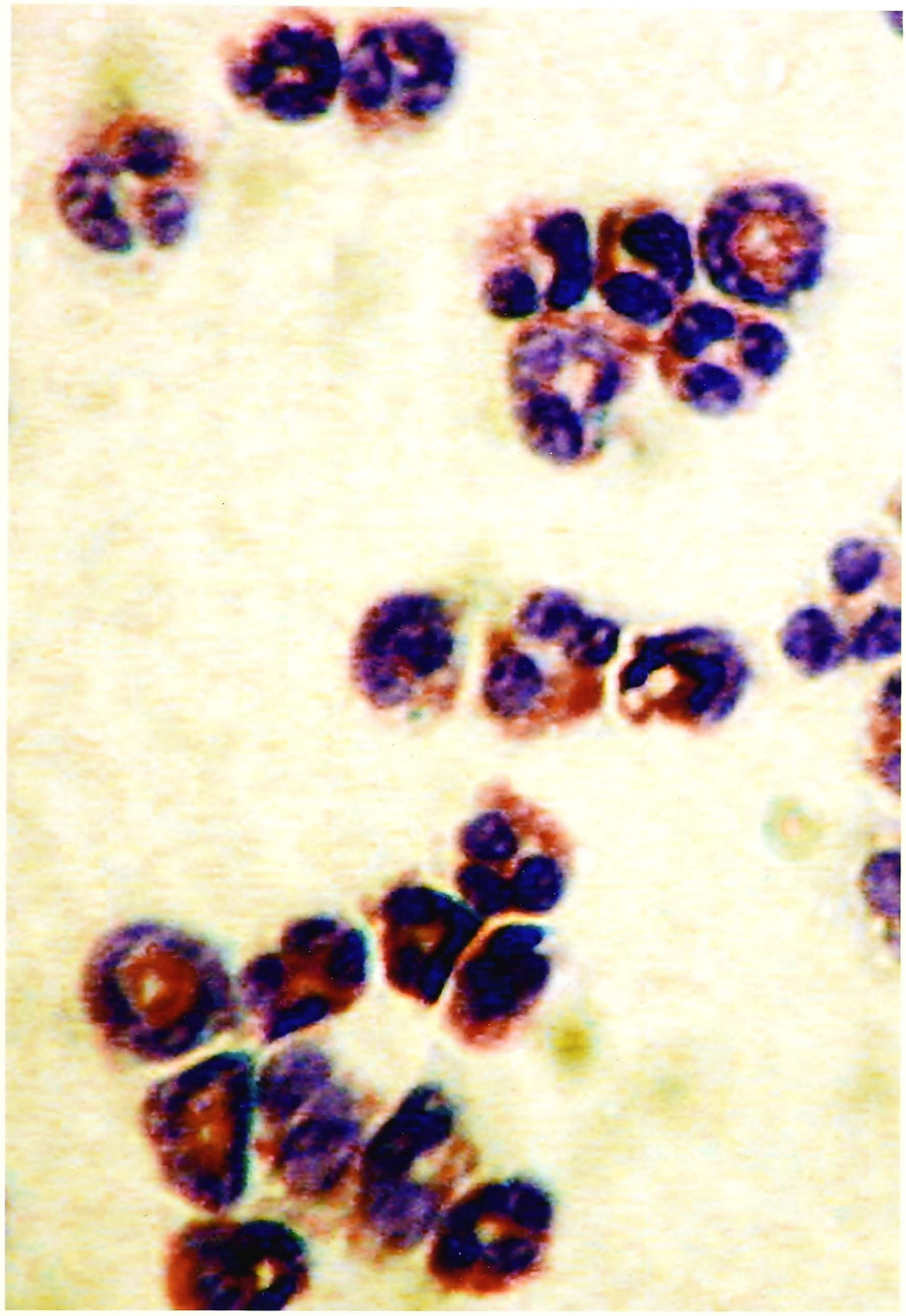


FIGURE 3.5- Eosinophils are the leukocyte population with high side scatter and light polarization properties. Cells were purified by FACS using the selection criteria established in previous experiments (see Fig. 3.3 and Fig. 3.4). To identify the leukocyte population, the purified cells were cytopsun on glass slides and stained using the May-Grunwald-Giemsa stain as described in *Materials and Methods* (section 3.2.6).

corresponded to eosinophils (Fig. 3.5). Hence, by narrowing the gates established using the BALF sample, eosinophils from the bone marrow and blood could be obtained with very high purity (> 96%; see Table 3.2).

3.3.3 Eosinophil populations isolated by FACS were highly pure, viable and responded to exogenous stimuli

The quality of the purification process was assessed by monitoring the viability of eosinophils using the trypan blue exclusion assay, differential counts of cytospins and response of eosinophils to PMA. Eosinophils isolated by FACS had a purity in excess of 96% and the viability within these populations was also very high (>95%) (Table 3.2). The predominant leukocyte contaminating the eosinophil population was the neutrophil (Table 3.2). Unfortunately, the yield was significantly affected in the bone marrow and blood samples since the gates had to be carefully selected to exclude as many neutrophils as possible.

In the previous Chapter, it was demonstrated that eosinophils in the BALF were highly activated and a small fraction were undergoing apoptosis. Thus, although the cells are viable according to the trypan blue exclusion assay, the cells may be no longer responsive and/or at early stages of cell death. Since it was established that eosinophils originating from IL-5 Tg mice respond to PMA (Chapter 2), BALF eosinophils were assessed for similar properties. Notably, BALF eosinophils adhered and degranulated after stimulation with PMA (Fig. 3.6).

3.3.4 Purification of total RNA from mouse eosinophils

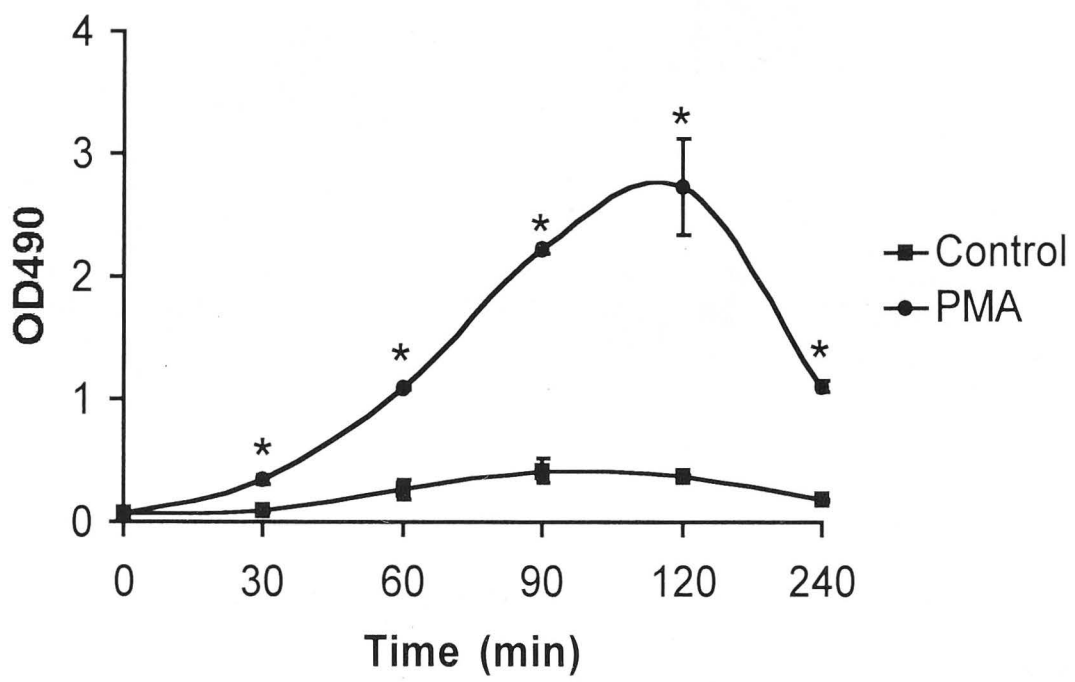
Since the objective of these experiments was to identify differentially expressed genes within eosinophils as the cells migrate to sites of allergic disease, intact RNA must be isolated to prevent artefactual results. Hence, all RNA samples were subjected to gel electrophoresis to determine the quality of the material. The traditional one-step solutions permitted the extraction of good quality RNA from total bone marrow, blood and BALF cells. Furthermore, total RNA could be isolated from cells after a 6 hour incubation on ice (the time required for the purification of eosinophils; Fig. 3.7). Unfortunately, the RNA isolated from purified eosinophils was degraded. Several modifications to the extraction protocol were attempted to improve the quality of the RNA sample. By performing a second extraction, the quality of the transcripts was improved (Fig. 3.8). However, purification of cells directly into the one-step solution,

Table 3.2- Efficiency of purification method.

Source	Purity	Contaminants	% yield	Viability	% mature eos.
Bone Marrow	> 96%	neutrophils/ lymphoid progenitors	~ 20%	> 95%	~ 75%
Blood	> 97%	neutrophils	~ 10-20%	> 95%	100%
BALF	> 98%	macrophages	~ 50%	> 95%	100%

Cells from the bone marrow, blood and BALF of allergic mice were isolated using standard techniques. Subsequently, red blood cells were lysed in an ammonium chloride solution and the leukocytes were resuspended in PBS containing 1% fetal calf serum. Eosinophils were purified from each sample by FACS using the high SSC and light polarization properties of this leukocyte population.

A Adhesion



B Degranulation

FIGURE 3.6- Purified eosinophils were activated upon PMA stimulation. Eosinophils isolated from BALF were cultured in the presence of 10^{-7} M PMA or vehicle control as described in the *Materials and Methods*. A. Cell adhesion. At each time point, the wells were washed with warm PBS and adherent cells were lysed with a 0.22% CTAB solution. A colorometric assay was employed to quantify the levels of EPO in each fraction. The data is presented as the average OD_{490} units ± SEM from three samples. Significant differences are $*p < 0.05$. B. Degranulation. After a 4 h period of stimulation, the supernatant was recovered and extracellular MBP was detected using an immunodot blot. A representative blot from 3 experiments is presented.

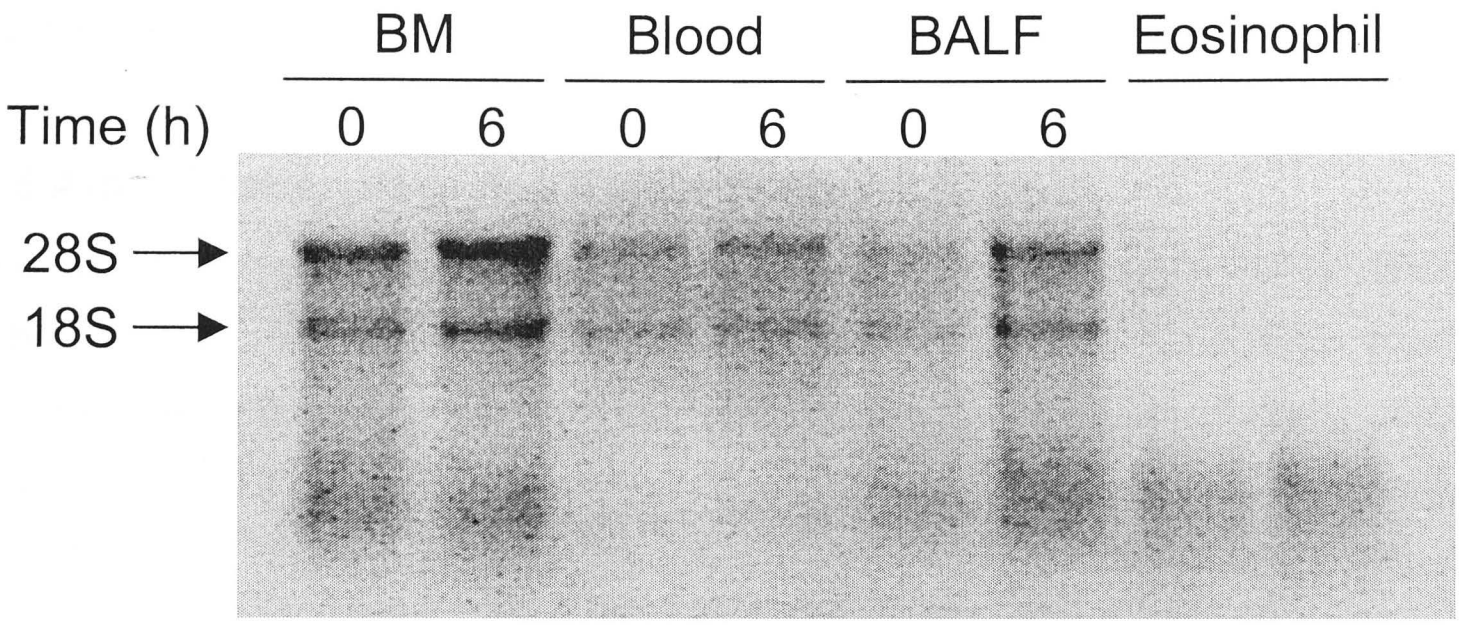


FIGURE 3.7- Extraction of intact RNA from total cellular populations in the bone marrow, blood and BALF. Cells were isolated from the bone marrow, blood and BALF as described in the *Materials and Methods*. Subsequently, the cells were either lysed immediately or incubated on ice for 6 hours before lysis in RNAzol B. RNA was extracted from total cellular populations or purified eosinophils using RNAzol B according to the manufacturers' protocol. Approximately 1-2 μg of RNA from each sample was loaded onto a 1.2% formaldehyde-agarose gel, stained with ethidium bromide and visualized using a UV transilluminator.

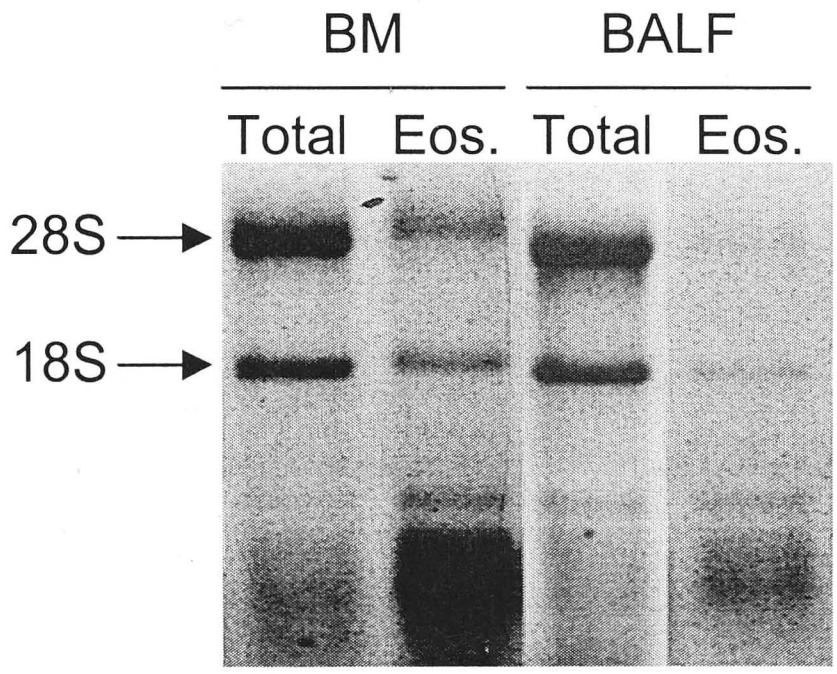


FIGURE 3.8- Optimization of RNA extraction from eosinophils. RNA was extracted from total bone marrow or BALF cellular populations, or purified eosinophils using RNAzol B. The extraction procedure was performed twice as described in the *Materials and Methods*. Approximately 1-2 μg of RNA from each sample was loaded onto a 1.2% formaldehyde-agarose gel, stained with ethidium bromide and visualized using a UV transilluminator.

purification of cells over a short period of time, treating cells with RNAlatter^{TM} or isolation of cells from IL-5 Tg mice did not improve the quality of the RNA.

3.3.5 Amplification of genes from eosinophil RNA

Since RNA which depicts a 2:1 ratio of the 28S/18S bands by gel electrophoresis could not be isolated from eosinophils, it might be a consequence of the large quantities of ribonucleases in the cell. However, the RNA may still be useful in studying differentially expressed genes. To further characterize the RNA samples, reverse transcriptase (RT)-PCR was performed using RNA isolated from total BALF cells and purified eosinophils. In both circumstances, transcripts predominantly expressed by eosinophils including MBP, CCR-3 and IL-5Rα could be amplified (Fig. 3.9). The results suggest that a fraction of the RNA remains intact but does not exclude the possibility that the quality of the RNA varies significantly from batch to batch or between different eosinophil populations. A semi-quantitative approach was performed to assess this latter possibility. GAPDH was amplified from control RNA, RNA isolated from bone marrow eosinophils and BALF eosinophils. Although significantly less GAPDH transcript was present in the eosinophil RNA samples compared to the control RNA, the levels of GAPDH were equal between all eosinophil-derived samples (Fig. 3.10). Thus, the RNA degradation is consistent between samples or the RNA profile obtained by gel electrophoresis represents the actual RNA content of eosinophils.

3.3.6 Several genes are differentially expressed between bone marrow and BALF eosinophils

After extracting RNA from bone marrow and BALF eosinophils, semi-quantitative RT-PCR was employed to identify if certain genes were differentially expressed in these cell populations. The RNA quality was verified by amplifying GAPDH and the levels of all other transcripts were normalized against GAPDH. Mouse eosinophils express cytokines and their receptors, chemokine receptors and cationic proteins. The transcription of these genes was greater in the BALF population except for MBP which was highly expressed in differentiating eosinophils (Fig. 3.11). Interestingly, IL-13, the IL-4Rα and IL-13Rα_1 subunits were highly expressed in BALF eosinophils.

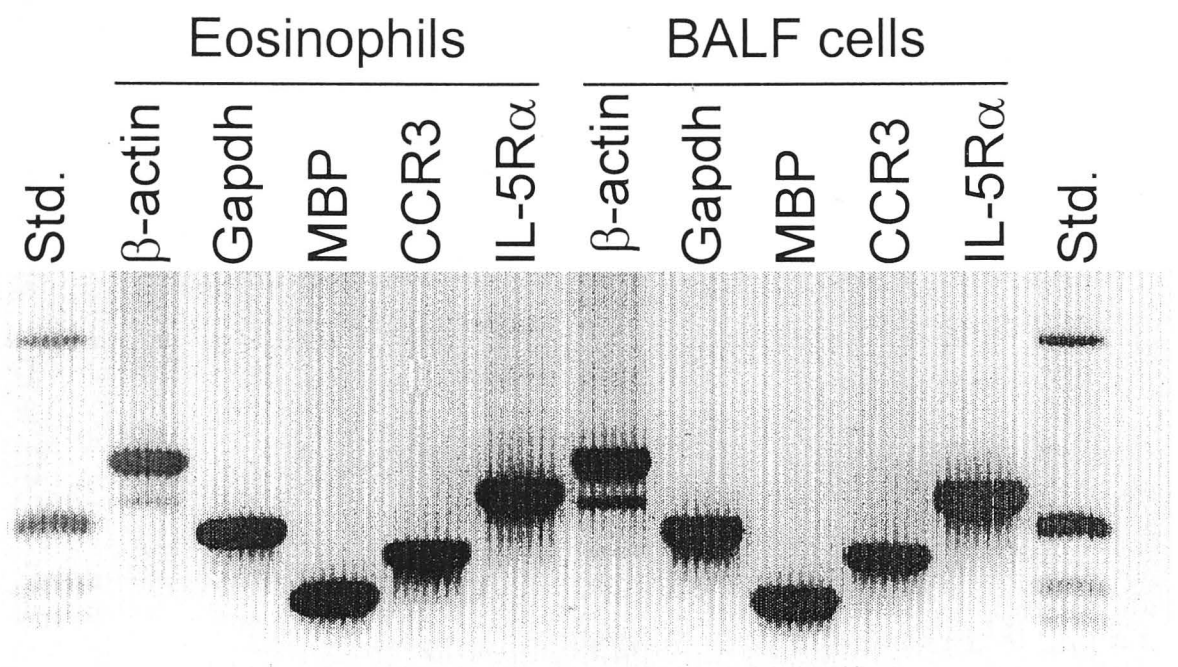


FIGURE 3.9- Genes predominantly expressed by eosinophils can be amplified by RT-PCR. RNA (1 μg) extracted from total BALF cells and purified eosinophils was subjected to first-strand cDNA synthesis followed by 35 cycles of PCR amplification. The PCR products were resolved on a 1% agarose gel, stained with ethidium bromide and visualized using a UV transilluminator.

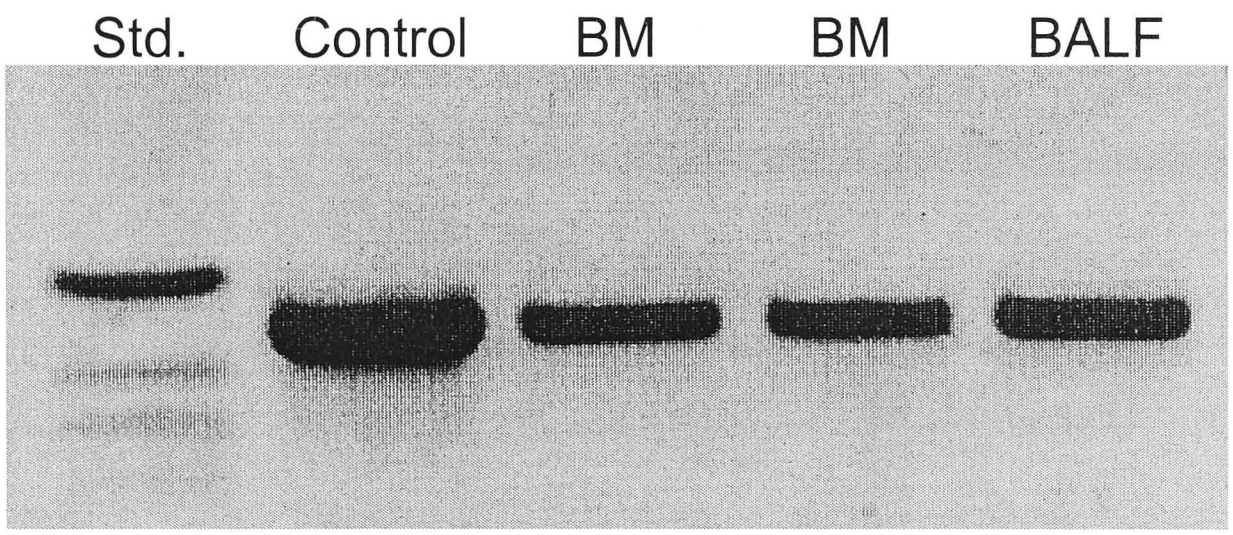


FIGURE 3.10- GAPDH levels are equal between bone marrow and BALF eosinophils. First-strand cDNA was generated from 1 μg of total RNA. Subsequently, GAPDH was specifically amplified by PCR. The amount of PCR product was analyzed on a 1% agarose gel after 20, 25, 30 and 35 cycles. A representative image of the PCR products after 25 cycles is presented.

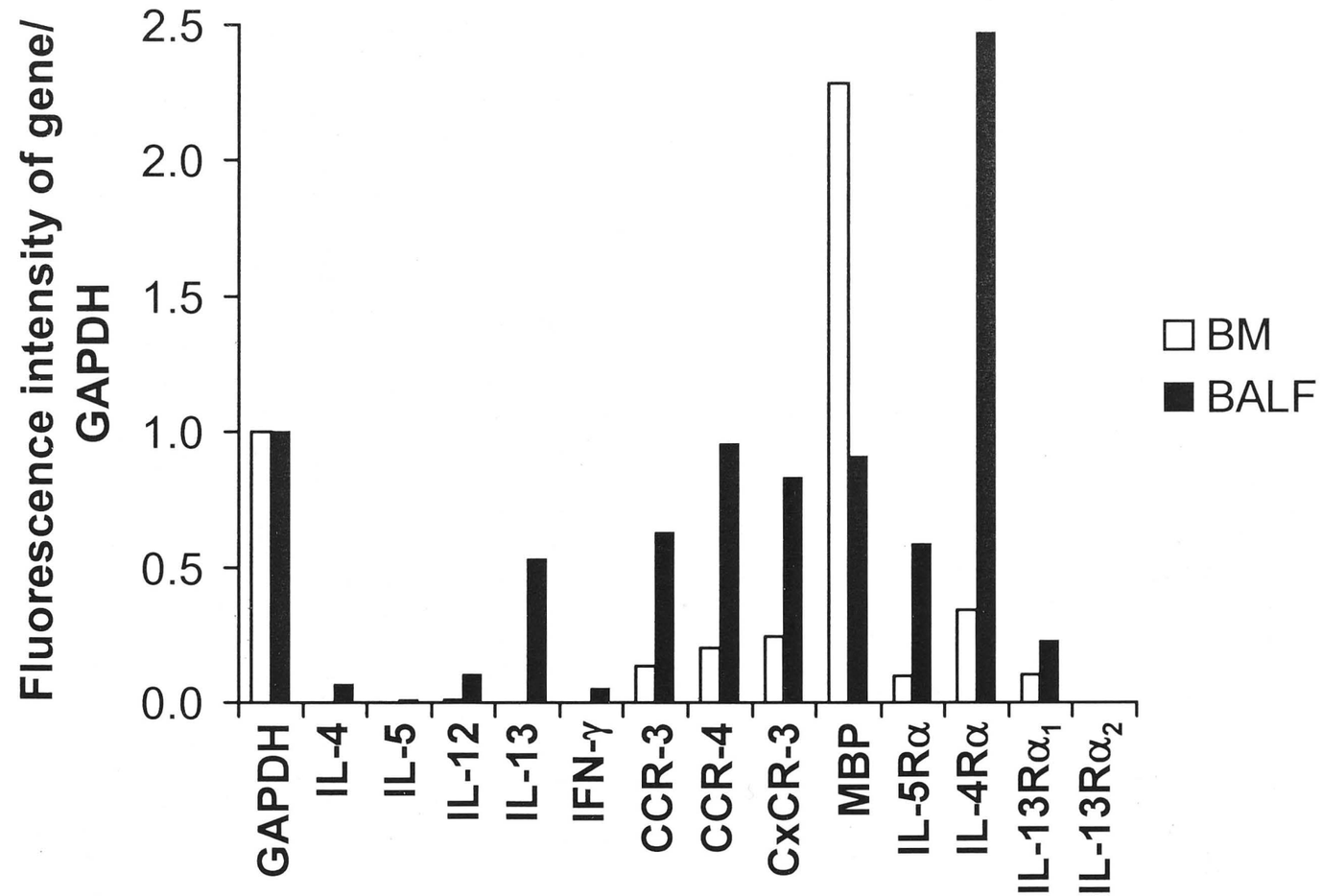


FIGURE 3.11- Cytokines, cytokine and chemokine receptors and granule proteins are differentially regulated between bone marrow and BALF eosinophils. Total RNA was extracted from purified eosinophils from the bone marrow and BALF. Subsequently, 1μg of RNA was subjected to RT-PCR using gene specific primers. The PCR products were separated on a 2% agarose gel and visualized using SYBR green. The signal intensity was measured using a Fluorescent Image Analyser FLA-3000. The data is reported as the ratio of fluorescence intensities of specific genes and GAPDH.

3.4 DISCUSSION

Mouse models of allergic airways disease, in combination with the use of genetically modified mice, has allowed the dissection of signals regulating the differentiation and recruitment of eosinophils during inflammation. However, a more detailed analysis of the mechanisms regulating eosinophil functions has been hampered by the lack of adequate methods to purify different eosinophil populations. In this Chapter, the high SSC and light polarization properties of eosinophils were utilized to develop a FACS-based purification method. The procedure permitted the purification of eosinophils resident in the bone marrow, blood and airway lumen of allergic mice. Furthermore, the cells were viable and responded to stimuli. This method may serve as a powerful tool to further study eosinophil biology in collaboration with the mouse models of asthma.

The FACS-based method overcomes the primary limitation of the other mouse eosinophil purification procedures, which is the lack of a marker to distinguish granulocytes. Light polarization properties of cells is an adequate criteria to differentiate each leukocyte population including granulocytes. Neutrophils polarize light significantly less than eosinophils. The difference may be attributable to the crystal core present in the secondary granules of eosinophils. Unfortunately, the neutrophil and eosinophil populations slightly overlap on FSC vs. SSC and light polarization plots. To discriminate between the two leukocytes, it is necessary to select narrow gates which allow for the selection of a specific cell type. While this strategy results in highly pure samples (> 96%), low yields are obtained. Another potential disadvantage may arise from this process as the purified population may not represent the diverse phenotypes of eosinophils in the original sample but be a sub-population. Hence, additional experiments aimed at the identification of other selection markers to distinguish granulocytes could improve the yield provided by this purification method.

During the preparation of this manuscript, another method has been described for the purification of mouse eosinophils from the blood of IL-5 Tg mice (Borchers *et al.*, 2002; Shen *et al.*, 2003). The procedure involves the separation of the cells first by a density gradient followed by the depletion of T- and B-cells using immunomagnetic beads. This method permits the isolation of $5x10^8$ eosinophils from 2 ml of blood. This method may provide an alternative to the FACS-based purification procedure. However, the density of eosinophils is dependent on the activation status of the cell (Busse *et al.*, 1996). Therefore, in samples where eosinophil activation is high (generating a hypodense population), the purified eosinophils may represent a sub-population. In addition, the bone marrow contains many immature eosinophils which will also have a variable

density. Hence, the density of the gradient will have to be carefully optimized to permit the maximum recovery of eosinophils.

The FACS-based purification method possesses certain advantages which include ease of sample preparation, reproducible sample purity, the method does not require the ligation of a cell-surface marker with an antibody and it can be applied to many sources of eosinophils. Furthermore, the procedure may also be used as an identification tool rather than a purification method. The alternative approach may be used to rapidly screen for differences in cell surface markers on eosinophils. It should be noted that lymphocyte, neutrophil and macrophage populations can also be analyzed in a single experiment. Although not indicated on the FACS plots, all leukocytes can be distinguished from one another.

The advantages provided by this purification method make it an ideal procedure to compare the phenotypes of different leukocyte populations. Firstly, the preferable purification methods are based on a negative selection procedure because they do not require the ligation of a cell-surface marker with an antibody on the cell under selection. This prevents the introduction of artefactual results due to the potential activation of intracellular signaling cascades in response to the binding of the antibody to a receptor. The FACS-based method avoids this problem as it takes advantage of physical properties of each leukocyte population. Furthermore, only one purification method is required to isolate eosinophils from the bone marrow, blood and BALF which prevents the differences in phenotype being attributable to the isolation methods themselves. Importantly, the purification method results in the isolation of viable and stimulus-responsive eosinophils suggesting that the stresses imposed by the purification method are not harmful to the cells. Collectively, the analysis suggest that the purification method will permit an accurate comparison of the different eosinophil populations.

The method was developed to study phenotypic differences within eosinophil populations as they migrate from the bone marrow to sites of inflammation. An initial screening procedure, called subtractive hybridization, was to be employed to identify mRNA transcripts which are differentially expressed in the eosinophil populations. The results in the previous Chapter suggest that comparison of bone marrow and BALF eosinophils should permit the identification of genes involved in the differentiation, migration and activation of eosinophils. Due to the high sensitivity of subtractive hybridization, intact RNA must be isolated to prevent artefactual results. Unfortunately,

initial attempts to isolate total RNA from mouse eosinophils failed. These results might have been due to the high concentrations of RNAses in eosinophils. Both EDN and ECP possess ribonuclease activity (Domachowske *et al.*, 1998a; Domachowske *et al.*, 1998b). In order to circumvent these problems, multiple modifications to the procedure were attempted to optimize the method. While performing a second extraction with the one-step solution enhanced the purity and quality of the RNA, all other modifications failed to further improve RNA quality. Importantly, total RNA which was isolated from eosinophils with the best extraction method did not display the traditional band pattern upon gel electrophoresis (28S/18S ration). It is unknown whether the pattern observed for total RNA from eosinophils represents the status of RNA in the cell or is due to partial degradation of the RNA during the extraction procedure. However, analysis of all the collected data suggests that the first proposition might be correct. Firstly, the RNA isolated from the total leukocytes present in the bone marrow and BALF display the appropriate intensity ratio between the 28S and 18S bands. Thus, very little degradation occurs during the isolation of total RNA most likely because the one-step solution stabilizes the RNA and inhibits eosinophil RNases. The material near the bottom of the gel might be degradation products of total RNA. However, it must occur in the cells before lysis. Furthermore, since this band is amplified in the eosinophil sample, it may be originating from these cells. It could be the result of cells which are actively undergoing apoptosis or cell death. However, viability of cells in all samples was always very high. Finally, RT-PCR analysis of GAPDH demonstrated that the levels of this gene are equal between bone marrow and BALF eosinophils. Moreover, the results were reproducible between different RNA samples which were isolated on separate occasions. The data strongly suggests that the total RNA extracted from eosinophils represents the conditions within the cell. However, it cannot be excluded that degradation of the RNA occurs at various stages of the experimental protocol.

Preliminary experiments demonstrated that subtractive hybridization might lead to the discovery of many genes. By means of semi-quantitative RT-PCR, several cytokines, cytokine and chemokine receptors were determined to be differentially expressed between the bone marrow and BALF eosinophils. A representative subset of genes from each family were initially analyzed. These genes were selected since they play an important role in eosinophil biology and/or have been implicated in the pathogenesis of allergic airways disease.

Eosinophils express a number of Th1 and Th2-type cytokines (Woerly *et al.*, 1999). Although allergic diseases arise from a Th2-type immune response, activated

eosinophils in BALF induce the expression of cytokines regardless of the class of immune response with which they are associated. Hence, not only is the expression of IL-4, IL-5 and IL-13 upregulated in BALF eosinophils but also, IL-12 and IFN-γ. The significance of the increase in expression of both Th1 and Th2-type cytokines in activated eosinophils during a Th2 immune response is unclear.

IL-5 is involved in the differentiation of eosinophils (Sanderson *et al.*, 1985) and signals via the IL-5Rα subunit in complex with a common β subunit of the IL-3, IL-5, GM-CSF receptors (D'Andrea and Gonda, 2000). Interestingly, an increase in mRNA levels of the IL-5Rα gene was detected in activated eosinophils. This result is unexpected but may reflect the importance of IL-5 signaling in priming the eosinophils for chemotaxis and activation (Carlson *et al.*, 1993; Fujisawa *et al.*, 1990; Kita *et al.*, 1992; Sehmi *et al.*, 1992; Takafuji *et al.*, 1996; Warringa *et al.*, 1992) along with serving as a survival signal (Yamaguchi *et al.*, 1991).

Various chemokine receptors previously reported as expressed on the surface of eosinophils were also up-regulated in BALF eosinophils (Jinquan *et al.*, 2000; Rothenberg *et al.*, 1999). Notably, human eosinophils undergo chemotaxis in response to MCP, a ligand of CCR-4 (Bochner *et al.*, 1999). However, the authors were unable to detect CCR-4 mRNA or protein expression in human eosinophils (Bochner *et al.*, 1999). In agreement with the human study, Borchers and colleagues (2002) could not detect CCR-4 expression in IL-5 Tg mouse eosinophils by RT-PCR. In contrast, we found that mouse eosinophils isolated from the bone marrow and BALF of allergic mice express CCR-4 mRNA with the transcript up-regulated upon activation of the cell. The differences between the two studies may be due to the source of eosinophils and/or the primer sets used for RT-PCR. To settle the discrepancy, detection of CCR-4 transcripts in mouse eosinophils from the different sources should be performed by Northern blot. Although eotaxins are believed to strongly contribute to the migration of eosinophils into tissue, ligands for CCR-1, CCR-3, CCR-4 and C-x-C chemokine receptor (CxCR)-3 may also contribute to the process of eosinophil recruitment.

Emerging evidence has identified IL-13 as a key regulator of allergic airways disease. This cytokine modulates several biological functions associated with allergic diseases including the recruitment of inflammatory cells, mucus hypersecretion and AHR (Grunig *et al.*, 1998; Matsukura *et al.*, 2001; Moore *et al.*, 2002; Terada *et al.*, 2000; Venkayya *et al.*, 2002; Walter *et al.*, 2001; Wills-Karp *et al.*, 1998). Both IL-4 and IL-13 initiate signaling cascades within a cell by means of a common IL-4Rα chain (Callard *et al.*,

1996). This receptor component must heterodimerize to form an active receptor. In the case of IL-13, two receptor chains regulate IL-13 signaling namely, IL-13Rα_1 and IL-13Rα_2 (Callard *et al.*, 1996; Kawakami *et al.*, 2001). However, recently new evidence suggests that IL-13 may signal via additional receptor systems independent of the IL-4Rα chain (Mattes *et al.*, 2001). With regards to eosinophils, IL-13 has been implicated in cell survival, chemotaxis and activation of this leukocyte (Dubois *et al.*, 1998; Horie *et al.*, 1997b; Luttmann *et al.*, 1996). Due to the emerging importance of IL-13, the expression of this cytokine and its receptors in eosinophils was investigated. Eosinophils can contribute to the pathogenesis of allergic diseases not only by secreting cytotoxic cationic proteins but maybe also by releasing IL-13. The expression of IL-13 was highly up-regulated in BALF eosinophils. Since IL-13 induces the production of eotaxin in the lung (Hirst *et al.*, 2002; Matsukura *et al.*, 2001), eosinophils may promote the recruitment of additional eosinophils by releasing this cytokine. In addition, eosinophil-derived IL-13 could promote AHR (Grunig *et al.*, 1998; Venkayya *et al.*, 2002; Wills-Karp *et al.*, 1998; Yang *et al.*, 2001). In agreement with previous functional assays (Dubois *et al.*, 1998; Horie *et al.*, 1997b; Luttmann *et al.*, 1996), subunits of the IL-13 receptor were constitutively expressed in mouse eosinophils. In particular, IL-4Rα and IL-13Rα_1 subunits were expressed in bone marrow at a basal level, which was increased in activated eosinophils but the IL-13Rα_2 subunit was not observed in either population. Further studying the role of IL-13 in the regulation of eosinophils may highlight novel roles of this cytokine in eosinophil biology.

Only one gene, namely MBP, displayed a higher expression level in eosinophils from the bone marrow compared to eosinophils present in the BALF, which is consistent with previous results using human eosinophils (Gruart *et al.*, 1992). MBP is one of the cytotoxic cationic proteins which are stored in secondary granules. Potentially, the other members of this protein family including EPO, ECP and EDN may also display a similar expression profile as reported for their human counterparts (Gruart *et al.*, 1992). The formation of secondary granules occurs primarily in progenitors and is complete in mature eosinophils (Popken-Harris *et al.*, 1998). Therefore, the expression of these genes would be expected to be highest in the bone marrow fraction as indicated by the results for MBP. However, this prediction cannot be made about all proteins involved in similar structures. Proteins associated with lipid bodies should be most highly expressed in BALF eosinophils since the number of lipid bodies increases upon activation of eosinophils (Bozza *et al.*, 1998).

The gene expression profile of MBP also provides additional information about the quality of the RNA. Degradation of the total RNA isolated from bone marrow eosinophils cannot explain the previous results obtained for the expression of cytokines, cytokine and chemokine receptors because other genes, in particular MBP, show a higher level of expression in the bone marrow sample. If the RNA was degraded, these differences would be lost or BALF eosinophils would display a higher gene expression. Collectively, the data provide strong support to pursue the generation and characterization of cDNA libraries by subtractive hybridization. The screening procedure may reveal the presence of a large number of differentially regulated transcripts and potentially, the discovery of novel genes involved in modulating eosinophil differentiation, migration and activation.

In conclusion, these investigations have provided a platform to further study eosinophil biology and compare the phenotype of various eosinophil populations. A method based on the light polarization properties of cells allows for the purification of viable and stimulus-responsive eosinophils from the bone marrow, peripheral blood and BALF of allergic mice. Furthermore, the extraction of total RNA from eosinophils has been optimized leading to the discovery of several differentially expressed genes using RT-PCR by bone marrow and BALF eosinophils. A complete characterization of the three eosinophil populations may provide additional insight into the molecular mechanisms regulating eosinophil differentiation, migration and activation.

4

General discussion and conclusions

4.1 DIFFERENTIAL REGULATION OF EOSINOPHIL DEGRANULATION BETWEEN SPECIES

During the last 5 years, a debate has emerged about the relevance of mouse models of allergic airways disease as experimental systems to study human disease processes. In particular, eosinophil degranulation, a key feature of the asthmatic lung, had not been demonstrated to occur in the lungs of allergic mice. This thesis has provided another perspective on this debate. While the data is in agreement with the previous studies depicting a low number or the absence of activated eosinophils in the lung submucosa (Malm-Erjefalt *et al.*, 2001; Stelts *et al.*, 1998), eosinophils are highly activated in the airway lumen. Furthermore, the proteins released into the airway cavity can promote pathogenesis of disease as they diffuse through the tissue to reach cellular targets. This observation is very interesting because Erjefalt and Persson (2000) have proposed that the infiltration by eosinophils into the airway lumen is a unidirectional pathway for the resolution of the tissue eosinophilia. However, this study in addition to several others (MacKenzie *et al.*, 2001; Shen *et al.*, 2003; Shi *et al.*, 2000), now suggest that while these migratory processes may serve as a mechanism for eosinophil clearance, they also permit the eosinophil to have a significant impact on disease.

One issue which remains unresolved, and could be the subject of future investigations, is the reasons for the different eosinophil phenotypes observed in the allergic lungs of humans and mice. Two plausible explanations can be hypothesized: 1) the micro-environment in the allergic lungs of humans and mice is significantly different and 2) eosinophil degranulation is differentially regulated between species. It would be difficult to address the first issue because our knowledge of the events leading to the pathogenesis of allergic diseases is yet to be fully defined. Novel genes which may encode chemokines and cytokines are being discovered on a regular basis. These classes of molecules may play an important role in mediating Th2 immunopathological processes, thus contributing to the development of pathophysiological features of allergic diseases. However, new high-throughput technologies may assist in determining whether differences in the micro-environment of allergic lungs exist between species. For example, DNA micro-chip analysis and/or application of proteomics (2D gels/ mass spectrometry) to this question would allow to identify the mRNA transcripts and the proteins which are differentially expressed in the allergic lung of mice and humans.

Although a more accurate reproduction of the micro-environment observed in the asthmatic lung would be beneficial, it may not necessarily lead to eosinophil degranulation in the lung submucosa of mice. The mechanisms regulating eosinophil activation may be very distinct between species. A previous study revealed that important differences in the response to exogenous stimuli exist between human and mouse eosinophils (Malm-Erjefalt *et al.*, 2001). However, the nature of these differences was never characterized. This thesis extends our knowledge with regard to these differences. Although the kinetics of adhesion and degranulation were conserved, mouse eosinophils required higher concentrations of exogenous stimuli to trigger cellular activation than human cells (Horie and Kita, 1994; Reimert *et al.*, 1998). This difference may contribute to the low level of eosinophil activation observed in the allergic lung of mice.

This basic difference in eosinophil activation may only be one of several differences. Another important question which remains unresolved regards the identification of physiological stimuli, which induce eosinophil degranulation. Do mouse eosinophils respond to the same stimuli as human cells? From the data in this thesis, we can conclude that at least some elements are preserved because mouse eosinophils also secreted MBP upon stimulation with antigen (OVA). OVA was selected as a possible candidate for inducing activation because antigen affects human eosinophil activation (Kaneko *et al.*, 1995b; Tomassini *et al.*, 1991), mouse eosinophils interact with OVA and present this antigen through MHC-II (MacKenzie *et al.*, 2001; Shi *et al.*, 2000) and OVA is a prominent molecule in the airway lumen during allergic airways inflammation. Interestingly, signals from T-cells in association with cognate antigen also appear to control eosinophil degranulation during parasitic infections in mice (Shinkai *et al.*, 2002). The results suggest that the reproduction of the conditions present in the airway lumen may enable the identification of other physiological stimuli activating eosinophil degranulation. Methods exist for the isolation of tracheal epithelial cells (Kumar *et al.*, 1997). By co-culturing these cells in conjunction with eosinophils in a media generated from activated Th2 lymphocytes, the effect of a wide range of molecules could be analyzed at once. Another approach to the problem would be to compare the stimuli present in the lung submucosa during allergic inflammation with those present in the airway lumen, since eosinophil degranulation does occur in this latter compartment.

While a tissue eosinophilia is associated with allergic diseases, the physiological role of eosinophils is believed to reside in host defense against parasite infections (Behm and Ovington, 2000; Meeusen and Balic, 2000). Comparing the response of eosinophils to

signals emitted during a parasitic infection and allergic disorders could provide another alternative method to further study mouse eosinophil activation.

Unfortunately, the current methods developed to study eosinophil activation *in vitro* have several limitations. The method reported in this thesis provides only a semi-quantitative analysis. Thus, the greatest challenge is to develop a better assay which would allow quantification of the level of secreted granule proteins. This methodology would increase the sensitivity of the degranulation assay and allow the detection of lower levels of degranulation. The immunodot blot is very insensitive and will only permit the detection of large quantities of MBP. Adapting the EPO detection method to the degranulation assay was attempted. However, it was discovered that the enzyme binds to the matrix used to coat the well and is unstable at 37°C (data not shown). The need for a sensitive assay is highlighted by the *in vitro* data collected from human eosinophils. Eosinophil degranulation does not appear to be a primary response to most stimuli as long periods of time (4 hours) are required to achieve full activation and only a small fraction (5-20%) of the total amount of protein available is secreted into the environment (Abu-Ghazaleh *et al.*, 1989; Fujisawa *et al.*, 1990; Horie *et al.*, 1996; Horie and Kita, 1994; Kaneko *et al.*, 1995b; Kroegel *et al.*, 1989; Takafuji *et al.*, 1994).

4.2 EOSINOPHIL PURIFICATION FROM BONE MARROW, BLOOD AND BALF OF ALLERGIC MICE BY FACS

The final contribution of this thesis to understanding eosinophil biology is the development of a method to purify eosinophils from the bone marrow, blood and BALF of allergic mice. To date, most methods only permit the isolation of mouse eosinophils from one compartment (Rothenberg *et al.*, 1995; Shinagawa and Anderson, 2000; Teixeira *et al.*, 1997). This limitation arises from the inability of the purification strategy to differentiate the granulocytes from one another. In order to purify eosinophils, the light polarization properties in conjunction with the SSC of cells were selected as criteria to separate all leukocyte populations including the granulocytes. This FACS-based method overcomes the primary limitation of other purification strategies and permits to differentiate neutrophils and eosinophils. This new method will permit comparative studies using functional assays, DNA micro-chip analysis and proteomics to identify phenotypic differences between each eosinophil population. In the long term, these future investigations could reveal key regulators of eosinophil differentiation, migration and activation which may serve as drug targets for novel therapeutics of allergic diseases.

A major benefit of this purification method is that a single procedure is utilized to isolate eosinophils from each compartment. Under these circumstances, all cells would be subjected to similar stress during purification. Furthermore, the ligation of an antibody to an antigen present at the surface of eosinophils is not required. This prevents the non-specific activation of receptor systems, which could alter the phenotype of the cells. Therefore, this procedure, most probably, results in eosinophil populations displaying gene expression profiles representative of the outcome of the multitude of signals present *in vivo*.

The method also permits the isolation of viable and stimulus-responsive cells. In this thesis, only PMA was utilized as a control to assess this feature of the eosinophil populations. In the future, a more detailed analysis of the response of eosinophils isolated from the various compartments to physiological stimuli may highlight significant phenotypic differences.

Unfortunately, the method has certain limitations. The most important one is the low yield especially in samples containing high neutrophil numbers. Although eosinophils polarize light significantly more than neutrophils, the profile on FSC vs. SSC and light polarization plots slightly overlap. Therefore, in order to obtain a high sample purity, it is necessary to apply very narrow gates which exclude all neutrophils and allow for the selection of eosinophils. A significant disadvantage may arise from this process as the purified population may not represent the diverse phenotypes of eosinophils in the original sample but may be a sub-population. However, the yield of eosinophils can be increased but to the detriment of the purity.

Future investigations may be pursued to improve the eosinophil purification strategy by adding an additional selection criteria which would further distinguish the neutrophil and eosinophil populations. Certainly, a positive selection marker such as the expression of CD49d, CCR-3 or IL-5Rα on eosinophils would have improved the yield and potentially, the purity. On the other hand, the ligation of the receptor with an antibody may change the overall phenotype of the eosinophil. Hence, the best option to avoid this manipulation of the cells is a negative selection marker for eosinophils. Human neutrophils are separated from eosinophils on the basis of differential expression of CD16 (Gopinath and Nutman, 1997; Hansel *et al.*, 1991b). Unfortunately, this procedure cannot be adapted for the isolation of mouse eosinophils because of two major limitations: 1) mouse eosinophils constitutively express CD16 (de Andres *et al.*, 1994) and 2) the only antibody available which detects mouse CD16, also detects

CD32 which is highly expressed on mouse eosinophils (de Andres *et al.*, 1994; Serrander *et al.*, 2000). By developing an antibody, which recognizes an epitope present specifically on neutrophils, would significantly enhance the purification procedure. Finally, a density gradient may be suitable to separate both granulocyte populations (Borchers *et al.*, 2002; Shen *et al.*, 2003). However, this procedure may lead to the preferential purification of an eosinophil population, especially in samples where eosinophil activation is very high, because activated eosinophils are hypodense.

A comparison by semi-quantitative RT-PCR of the expression of a selection of genes involved in the pathogenesis of allergic diseases revealed that granule proteins, cytokines and their receptors and chemokine receptors are differentially expressed between bone marrow and BALF eosinophils. Notably, IL-13 is highly expressed in BALF eosinophils. Furthermore, the IL-4Rα and IL-13Rα_1 transcripts are up-regulated in activated eosinophils. Since IL-13 has been recently shown to play an instrumental role in the pathogenesis of allergic airways disease (Grunig *et al.*, 1998; Mattes *et al.*, 2001; Walter *et al.*, 2001; Wills-Karp *et al.*, 1998), eosinophils may respond to IL-13 signaling as well as transmit IL-13 -based signals to affect disease processes. Based on these limited results, future research should focus on screening the genome for differentially regulated genes in eosinophil populations using DNA micro-chip technology or subtractive cloning. These experiments may highlight novel mechanisms utilized by eosinophils to affect the pathogenesis of allergic diseases.

4.3 CONCLUDING REMARKS

This thesis has provided new insights into the role of eosinophils in mouse models of allergic pulmonary inflammation and new methods to further investigate the mechanisms regulating eosinophil activation. Additional research which aims at understanding the molecular mechanisms of mouse eosinophil degranulation in collaboration with comparative studies of the micro-environment in the allergic lungs of humans and mice will permit the development of improved mouse models of allergic airways disease. These models may be helpful in the development of novel therapeutics for allergic diseases.

5

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